

For the want of a test...

As Europe wrestles with its BSE crisis, the top priority is to develop a diagnostic test that can reliably identify animals incubating the disease and people incubating its human form.

“We are in the midst of an epidemic of fear,” said James Mason, director of the US Centers for Disease Control, in 1985. He was referring to the public’s reaction to AIDS, then a newly emergent disease.

Fast-forward to 2001, and there is another epidemic of fear. Over the past few months, it has become clear that bovine spongiform encephalopathy (BSE) is a pan-European problem. Countries such as Germany, Italy and Spain, which for years regarded themselves as BSE-free, have been forced to admit that their cattle herds are harbouring the disease. Consumers feel betrayed, and sales of beef have plummeted across the continent. The shock wave is also being felt in the United States, where federal agencies are scrambling to prevent the disease crossing the Atlantic (see *Nature* **409**, 441–442; 2001).

But there is one important difference between AIDS in 1985 and BSE in 2001. In March 1985, the US Food and Drug Administration approved the first blood test for HIV infection. At a stroke, it became possible to screen donated blood for the virus. For those lucky enough to live in countries with well-regulated blood supplies, avoiding AIDS became primarily a matter of abstaining from risky sexual practices.

By contrast, there is still no reliable way to identify cattle incubating BSE, or people infected with its human form, variant Creutzfeldt–Jakob disease (vCJD). In an attempt to put some figures

to the BSE epidemic, the European Commission has instituted a programme of diagnostic testing of brain tissue from slaughtered cattle (see page 658). But while the tests being used may have some value in picking up preclinical cases, they have not been validated for this purpose. Unfortunately, the equivalent of the HIV blood test — a reliable diagnostic capable of identifying infected animals or people soon after they become infected — is not yet available.

For agencies confronting the BSE epidemic, producing such a test should be a top priority. Not only would it be a valuable epidemiological tool, but it would also offer a means to reliably exclude infected animals from the human food chain. And because potential therapies are likely to require intervention before symptoms appear, progress in diagnostics is also key to saving people who are incubating vCJD (see page 660).

But there is a danger that the validation of candidate diagnostic tests could become a bottleneck. It will require the tests to be put through their paces on tissue samples taken from animals that were deliberately infected but have yet to show any symptoms. Currently, the only source of such material is Britain’s Central Veterinary Laboratory in Weybridge, Surrey. To ensure that sufficient reference material is available, the European Commission should move urgently to create a central repository. ■

Handling (mis?)appropriated data

Introducing a policy to ensure due credit for unpublished data.

The practice of posting unpublished data in publicly accessible databases is widespread in the genome sequencing community. Given the years it can take to produce a finished sequence, such openness makes a significant difference to the rate at which science and its applications can develop. But there is a downside. Researchers who post data in this way may lose the opportunity to exploit them as others promptly seize the data and run with them. That is a (sometimes reluctantly) accepted consequence of openness. What is much more controversial is a refusal by the appropriators of posted data to give credit to the originators of those data.

Previously (see *Nature* **405**, 719; 2000), we stated some elementary principles in our approach to this issue. Briefly, such posting of unpublished data does not count as prior publication, but neither is it protected from appropriation and publication by others in any way, unless a licensing agreement is explicitly required. The latter approach has been adopted, for example, by The Institute for Genomic Research in Rockville, Maryland, whose licensing agreement (see <http://www.tigr.org/tdb/license.shtml>) requires users to agree not to use TIGR’s unpublished data for global genomic analysis before their publication of a complete genome paper.

We also urged more from the community by way of sensitivity to the interests of originators. Some sequencers have urged that *Nature* simply refuse to consider papers where inadequate credit is given. And it has recently been suggested that appropriators who do not obtain written consent from originators before making use of these

data in publications are by definition misappropriating the data and committing fraud (see Hyman, R. W. *Science* **291**, 827; 2001).

While we agree that seeking consent is important, we do not accept that using data if consent is refused necessarily constitutes fraud, as consent can sometimes be withheld for questionable reasons. However, there is also a need to ensure that appropriated data are being used in full awareness of their technical limitations, given that they are sometimes preliminary or may be subject to qualifications that only their originators are fully aware of. And we do believe that practical steps can be taken to ensure that credit is given where, as far as it is possible for us to judge, we believe it is due.

Accordingly, we have decided to adopt the following practice. Appropriation of uncredited data will not prevent us from sending a paper out for prompt review. But we will require written assurance that authors are not violating any originators’ data-licensing agreement. We will encourage our referees to be alert to the use of appropriated unpublished data from databases. Where there are concerns over credit, we will usually seek advice from an originator of the data in addition to the usual refereeing process. We would not be giving originators a veto: where disagreements arise, we will use our judgement, having consulted referees over technical considerations if necessary, and will usually insist on an acknowledgement as a condition of publication. As with all policies, we shall keep this under review, and welcome the opinions of readers, which should be sent to nature@nature.com. ■





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Japan's ape sequencing effort set to unravel the brain's secrets

David Cyranoski, Tokyo

A workshop planned for next month on the genetics and neurology of apes could pave the way to a better understanding of the relationship between the human genome and the brain, its organizers say.

The Genes and Minds Initiative (GEMINI) workshop will try to improve coordination of existing ape-genome sequencing efforts and of related research in neurological gene expression and gene-based evolutionary study of apes. It could also serve as a springboard for a major international effort in the sequencing of ape genomes.

The meeting is being jointly organized by the National Institute of Genetics (NIG) and the Institute of Physical and Chemical Research (RIKEN) in Japan, and will take place in Tokyo. "The aim is to provide a place to discuss possible international and interdisciplinary cooperation on understanding what makes us human," says Yoshiyuki Sakaki of RIKEN's Genomic Sciences Center.

The ape genome is thought to be around



Close relative: as ape genomes are similar to our own, comparative analysis could pay dividends.

99% equivalent to the human genome. "By examining the subtle genetic differences relating to the nervous system and brain function, it will be possible to shed light on

language, the ageing process, and on sensitivity to disease," says Hans Lehrach of the Max Planck Institute for Molecular Genetics in Berlin. For example, researchers might be able to establish what makes apes resistant to Alzheimer's disease and AIDS.

Sakaki and Lehrach hope to use the experience they gained sequencing chromosomes 21 and 22 for the human genome project in assembling ape genomes. Because of the great similarity, Lehrach thinks that they will be able to assemble many of the corresponding ape DNA fragments right on top of the human sequence. "This could reduce sequencing needs and make it a very cheap project, perhaps costing less than US\$100 million," says Lehrach.

Last year, RIKEN announced a project to compare chimpanzee and human genomic sequences, but commitment to the human genome and lack of funding have held back its progress (see *Nature* 406, 4; 2000). Now the NIG is involved, details of the plan are being firmed up — although funding is still elusive.

RIKEN's Brain Science Institute will provide the project with candidate genes, such as those related to language and brain development, from human brain research projects. These will be used to correlate genes to their function in the brain, using microarrays.

RIKEN's Genomic Sciences Center is

Science 'in crisis' says commission

Irwin Goodwin, Washington

Spending on science and technology in the United States should be doubled in the name of national security, says a report by a high-powered bipartisan panel.

The report, "Road Map for National Security: Imperative for Change", was prepared by the US Commission on National Security/21st Century. The commission, a 14-strong body established by Congress in 1998, is led by former senators Warren Rudman (Republican, New Hampshire) and Gary Hart (Democrat, Colorado).

Although the report contains scant evidence to support its assertion that US science and technology are "in crisis", its bombastic tone is likely to help drum up support in Washington for extra funding.

The commission's main thrust is to call for a complete overhaul of the defence department and civilian agencies such as the

Federal Emergency Management Agency. But it also urges President George W. Bush and Congress to double the funding of basic research and technology development — from the government's current annual investment of \$90 billion — by 2010.

It recommends that the president should give his science adviser more authority to set research objectives, and to coordinate the budgets of the 20 or so research agencies. The commission further suggests that Congress should pass a National Security Science and Technology Education Act, which would fund a programme to train more scientists and engineers and to produce better-qualified science and maths teachers for schools.

Rita Colwell, director of the National Science Foundation, which funds most non-biomedical research at US universities, described the report as "absolutely correct". ■

► <http://www.nssg.gov/phasesIIwoc.pdf>

expected to handle sequencing and initial analysis, starting with the chimpanzee equivalent of chromosome 21 and other genes suggested by the Brain Science Institute. The NIG will run a comparative study of chimpanzees, orang-utans, macaques and gorillas in an attempt to determine the way in which the various different species have evolved.

So far money for the project has not been forthcoming, with each group using funds from some of their other projects to allow them to do the planning. But Naruya Saito, a geneticist at the NIG, hopes that Japan is poised to take the leading role in an international ape sequencing project. He sees it as an opportunity for Japan to "show the world its sequencing capacity".

The project is also notable because it marks the first collaboration between two of Japan's premier research institutes under the auspices of the newly formed Ministry of Education, Culture, Sports, Science and Technology (see *Nature* **408**, 757; 2000). In the past, rivalry between the various agencies has made such a collaboration difficult. Gemini — the astrological sign of the twins — could therefore represent not only the relationship of genes and the mind, says Saito, but also a new match-up between the NIG and RIKEN.

► <http://sayer.lab.nig.ac.jp/GEMINI>

Japanese premier underlines opposition to human cloning

David Cyranoski, Tokyo

Japan's prime minister, Yoshiro Mori, has warned Japanese researchers to steer clear of a proposed international project to clone humans.

The project, led by Italian *in vitro* fertilization specialist Severino Antinori and University of Kentucky reproductive physiologist Panos Zavos, aims to help infertile couples have children using the same technology that has been used in animal cloning. The mother's egg would be injected with the father's genetic material and then implanted into her womb.

One project member has predicted that a baby will be born within two years using the technique. But, apart from strong ethical concerns, critics say that the technique's low expected success rate makes it unsuitable for human cloning.

Although many researchers and politicians have chosen to ignore the latest claims being made for the project, reports that a Japanese researcher is involved led to the prime minister's intervention.

A law passed last December, which comes into effect this June, outlaws human cloning in Japan. Mori said he was charging the Ministry of Education, Culture, Sports, Science



Seeing double: human cloning is a headache for Prime Minister Yoshiro Mori.

and Technology, and Takashi Sasagawa, the science minister, to take steps to keep Japanese researchers out of the project.

"We will try to persuade researchers that what they are doing is wrong and explain that it is a violation of Japanese law," says an official in Sasagawa's office. But participating in such a research project abroad would not directly violate Japanese law — although researchers might be reluctant to defy the government's wishes, and research agencies may cut their funding if they do.

Italian biologists left out in the cold

Alison Abbott

Italian research minister Ortensio Zecchino has sown discontent among biologists by excluding them from a key academic committee that he appointed just before



Zecchino's parting gift to life sciences was to snub them.

resigning from the government last week.

Zecchino did not include any life scientists in the new eight-strong 'warrant' committee, which will distribute annual grant money of around L250 billion (US\$120 million) to university researchers.

The committee includes two lawyers, a historian and an economist, as well as a chemist, an engineer, a mathematician and a paediatrician. Biologists feel Zecchino has abused his privilege of making the appointments directly, a privilege conferred only recently on the research minister.

In a letter to the newspaper *La Repubblica*, 14 Italian heads of scientific societies in the life sciences complained that their discipline was not represented on the committee, although biology accounts for more than a fifth of all grant applications.

"Italian universities require highly qualified evaluators of proposals in biological sciences," says the letter. It asks the prime minister, Giuliano Amato, who is acting as caretaker to the research ministry until the general election this spring, to add an appropriate expert to the committee.

Zecchino left the government to join a new political party, which is positioning itself to take part in the centre-right coalition that is expected to form the next government.

In a separate pre-election development, health minister Umberto Veronesi nominated biochemist Enrico Garaci to become president of the Istituto Superiore di Sanità, the national health institute, once the statute enabling the position is approved. Garaci is a previous president of the CNR, Italy's national research agency.

Germany targets international talent

Quirin Schiermeier, Munich

Germany is to offer a small number of elite overseas researchers grant support of DM4.5 million (US\$2.1 million) over three years to work in the country at an institution of their choice.

The Alexander von Humboldt Foundation, an independently administered body financed by the government, will offer between 15 and 20 awards to scientists with international reputations.

Although the main goal of these 'Wolfgang Paul' awards is to attract non-German scientists, native Germans are also eligible if they have been working abroad for more than five years.

Recipients will receive up to DM250,000 per year — considerably more than the salary of a full professor in Germany — for 'living costs'. The rest of the money can be used to finance research.

In addition, the foundation will offer 20 or 30 young scientists up to DM2.25 million each over three years.

► <http://www.avh.de/en/programme/neu.htm>

Greek plutonium haul raises smuggling fears

Quirin Schiermeier, Munich

The Greek authorities are stepping up investigations into the smuggling of nuclear material following the discovery of a cache of radioactive plates in the north of the country.

Around 350 plates containing a total of three grams of plutonium were unearthed last month in a forest near the city of Thessaloniki. But such a small amount is a long way from the several kilograms needed to build a nuclear weapon.

"The only explanation for their appearance is that they were smuggled into Greece by criminals hoping to find a gullible buyer," says Leonidas Kamarinopoulos, president of the Greek Atomic Energy Commission (GAEC).

The plates were found following an anonymous tip-off to police. They were taken to a nuclear research laboratory in Athens, where analysis by a GAEC team revealed the presence of plutonium-239 and traces of americium.

According to the Greek authorities, the mildly radioactive plates are probably machine components from eastern Europe. Kamarinopoulos believes that the plates were stolen from a paper plant, most probably in Russia, where devices containing plutonium are widely used as electrostatic eliminators. This technology is not used in the West.

But the circumstances of any planned

smuggling deal remain hazy. An anonymous informant apparently told the police that members of the Bulgarian mafia had hidden the material in the forest. But it is not clear whether the couriers were still arranging to sell the plates, or seeking to dispose of them after failing to find a buyer.

Although there have been several reported cases of attempted plutonium trading in Germany, Bulgaria and Turkey, this is the first known case of such smuggling in Greece.

Given the incident's rather amateurish circumstances, Kamarinopoulos is inclined to dismiss it as isolated case. Nonetheless, the Greek government is taking it seriously, and says it will explore the possible existence of a larger channel of nuclear smuggling activity through Greece.

According to David Kyd, a spokesman for the International Atomic Energy Agency (IAEA) in Vienna, the discovery is "a potential health hazard, but certainly not a serious proliferation risk". As nuclear weapons need several kilograms of plutonium, he says: "No interested party would buy plutonium in the magnitude of a few grams, hoping that the dealer will come back with more."

The IAEA has sent a safety expert to Greece to assist the local authorities in decontaminating the spot where the plates were found. Meanwhile, 200 cubic metres of soil have been removed, and all water

sources in the area have been sealed off. The vicinity is also being searched for any more radioactive material.

The IAEA has offered to help analyse the precise balance of plutonium isotopes in the material, which would help to track down its exact source.

Nuclear proliferation experts are cautious about judging the significance of the incident. "No matter how big the actual threat is, it is disturbing to see that some east European production and research facilities are not sufficiently safeguarded," says Spurgeon Keeny, president of the Washington-based Arms Control Association. ■



Top research universities face up to gender bias

Steve Nadis, Boston

The leaders of the top research universities in the United States are facing up to the fact that they have a problem with women.

Presidents, chancellors and provosts from nine elite institutions met up with 25 women academics last week at the Massachusetts Institute of Technology (MIT) to confront the issue of gender bias

in science and engineering.

Representatives from the schools — including the California Institute of Technology, Harvard, Princeton, Stanford and Yale — acknowledged that women researchers face substantial barriers to career advancement. Without promising any immediate specific actions, they vowed to work together to break these barriers down.

Biologist Nancy Hopkins, who has led the fight against discriminatory practices at MIT, describes the meeting as an "historic event—a milestone that I thought would never happen in my lifetime".

Harvard physicist Howard Georgi agrees. "The top institutions are the places where the [bias] problems may be the most serious because the faculty is the most competitive. If we can make inroads here, it will have an enormous impact," he says.

The participants pledged to go back to their institutions, collect information on factors such as hiring practices, salaries and committee membership, and then try to establish "a faculty whose diversity reflects that of the students we educate".

The group will reconvene in a year's time to collate their data and discuss what improvements, if any, have been made. The possibility of collective action will then be considered.

"The fact that we're all coming back a year from now creates some pressure to actually get something done," says MIT president Charles Vest, who organized the event. ■



Chewing the fat: Nancy Hopkins (centre) takes equality issues to university leaders at an MIT forum.

DONNA COVENEY/MIT

India struggles to cope with IT brain drain

K. S. Jayaraman, New Delhi

Scientific research in India is facing up to a brain drain with a difference. The financial lure of careers in information technology — at home and abroad — is creaming off more and more of the talented young people who might otherwise become scientists.

Addressing the Indian Science Congress last month, Prime Minister Atal Behari Vajpayee said the global demand for Indian computer professionals was a challenge for Indian science. Although the country was proud of its prowess in IT, Vajpayee said, he was worried about the decline in enrolment in other disciplines by the country's best students. He

warned that the trend could lead to a shortage of science teachers and researchers.

The state of Tamilnadu, one of the main sources of IT émigrés, reports a shortage of 500 university teachers, with few graduates choosing to go on to do a PhD, the entry-level qualification for institute faculty.

"All our best students are opting for IT," says one university administrator. "This is posing problems for our other faculties."

The head of the Defence Research and Development Organisation recently blamed a delay in developing a new fighter aircraft on a severe shortage of skilled staff. And many research institutes are feeling the impact.

"I would like to believe this is a passing phase and that things will stabilize sooner rather than later," says Govardhan Mehta, president of the Indian National Science Academy and director of the Indian Institute of Science in Bangalore, the country's leading research institute. Many science and engineering students at his institute have left for high-paying jobs in the IT sector, he adds.

Even India's own IT industry has been hit. "The attraction of the United States has led to a severe shortage of high-quality talent," says Naresh Gupta, managing director of Adobe India.

The government's main response has been to increase the supply of IT professionals, including teachers, announcing plans to expand undergraduate training and emphasize postgraduate and research programmes. It also plans to sponsor 200 students in all scientific disciplines to PhD level and upgrade 250 university science departments.

One estimate is that India will need to produce 1.5 million computer software engineers — half of whom will go abroad — during the next eight years.

Indian nationals are said to be involved in 40% of the start-up companies in California's Silicon Valley. The flow increased after the annual quota of US work permits for technically qualified immigrants went up from 107,500 to 195,000. Britain has tripled its quota to 90,000. Singapore, Japan and Germany are also taking steps to attract skilled immigrants.



Feed the world: Vajpayee (second from right) plans to step up supply of scientists and IT professionals.

NASA celebrates as spacecraft homes in on asteroid

William Triplett, Washington

Next Monday, 12 February, NASA scientists will be celebrating the success of the Near Earth Asteroid Rendezvous (NEAR) mission — even if the spacecraft crashes and disintegrates on the surface of the asteroid on which it will attempt to land.

This is because NEAR has already surpassed its original goals. The attempted landing, according to NASA spokesman Don Savage, is merely a chance to collect "bonus science" in the form of extremely high-resolution photographs that the spacecraft will take as it descends towards 433 Eros.

"Even if we just get one photograph," Savage says, "it will be more than we expected." There is an outside possibility that NEAR's instruments will survive a landing, because 433 Eros has only a weak gravitational pull. But NEAR was not designed for landing, and no one has tried landing a spacecraft on an asteroid before.

NEAR Shoemaker, as the mission is

formally known, was launched five years ago. After a two-billion-mile journey, it entered the orbit of 433 Eros, one of the largest near-Earth asteroids. The spacecraft then began collecting data for the first long-term, close-up study of an asteroid. It focused on mass, structure, composition, geology and magnetic field, in a search for clues to the origins of the Earth, the other planets, and possibly the entire Universe.

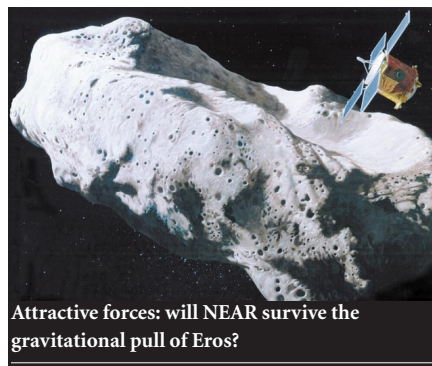
According to NASA, NEAR has collected 10 times more data than originally planned during its one-year orbit of 433 Eros, including indications that the asteroid is older than Earth. But some of the data have been mystifying, such as the disintegration of boulders on the asteroid's surface. Such questions prompted scientists at NASA and Johns Hopkins University's Applied Physics Laboratory, which built the craft and has managed the mission, to try for a closer look.

The success of the \$240 million mission marks a welcome contrast with the failures

and cutbacks in NASA's outer-planet programme during recent years.

Mark Sykes, chair of the American Astronomical Society's division of planetary sciences, supports the NASA plan: "Why just turn the thing off at the end when you can do something that's higher risk and you can gain from it?"

♦ <http://near.jhuapl.edu>



Attractive forces: will NEAR survive the gravitational pull of Eros?

Australian leader pledges research boost before election

Peter Pockley, Sydney

Australia's Conservative government — facing an election by the end of this year — has announced a package that it calls the nation's largest ever boost in research funding.

Revealed late last month by Prime Minister John Howard, the plan puts an extra A\$2.9 billion (US\$1.6 billion) into research over the next five years. It was welcomed by the science lobby as a change from the years of cuts that began with the government's first budget five years ago.

With its announcement, the government largely accepted the recommendations of year-long reviews of Australia's science base and capacity for innovation (see *Nature* 409, 123; 2001).

The new money became available under sustained pressure from industry, the universities and the opposition Labor Party.

A week before Howard's announcement, Labor leader Kim Beazley said that the pledge to rebuild the universities and expand research would be one of "four pillars" in Labor's election



Howard: came under pressure to stop making cuts.

campaign. He also promised to create an online university for up to 100,000 undergraduate students.

But the government's proposal will only allow for a slow phasing in of 20 programmes — half of them new — which have been singled out for expansion.

The first year, beginning this July, will see research spending grow by just 3.5% from its present level of A\$4.5 billion. And the overall five-year increase amounts to less than a third of the sum the 'group of eight' leading research universities says is needed.

Universities will receive most of the new funds, through grants from the Australian Research Council, investment in infrastructure, and two new 'centres of excellence' in biotechnology and information technology. The nation's largest research agency, the Commonwealth Scientific and Industrial Research Organisation, will not get any extra money from the package. ■

Tissue donations slump after revelations about misuse

David Adam, London

Donations of human tissue for research look set to slump in the United Kingdom following revelations about the systematic misuse of human remains at a Liverpool hospital.

A pathologist at Alder Hey Hospital was found to have stripped and stored every organ from every child whose parents allowed permission for a post-mortem. In the wake of a report on the episode by the Royal Liverpool Children's Inquiry, which was published last week, other pathologists are facing a crisis of public confidence that may lead to a shortage of donated tissue for research. The political fallout from the episode has also led to the immediate suspension of some research projects, with researchers unwilling to continue until ethical and legal guidelines are clarified.

The report focuses on the actions of former Alder Hey pathologist Dick van Velzen, Professor of Foetal Infant Pathology at the hospital between 1988 and 1995. It found that van Velzen systematically ordered the unethical and illegal stripping of every organ from every child who had a post-mortem.

The pathologist failed to tell parents that their children's organs would be retained. He claimed he needed the organs for research into sudden infant death syndrome, but the inquiry found that most were never used in research, or even properly examined.

Other pathologists have sought to distance themselves from his actions, but the incident has focused attention on organ collections and libraries of tissue samples held at hospitals and research centres across the country.

James Underwood, professor of histopathology at Sheffield University and vice-president of the Royal College of Pathologists, says that fewer people have agreed to organ and tissue removal since the news about Alder Hey hit the headlines in 1999.

"The number of hospital post-mortems is down and it is becoming increasingly difficult to get certain tissue for research, particularly brain tissue," he says. This is used to study conditions such as multiple sclerosis and variant Creutzfeldt-Jakob disease (vCJD).

James Lowe, a neuropathologist at the Queen's Medical Centre in Nottingham, says tissue shortages are already damaging his efforts to monitor vCJD cases in the region. "Pathologists are no longer confident about removing brain tissue samples during post-mortems," he says. "So if an elderly person with dementia falls and dies, for example, nobody is checking what disease may have caused them to fall." Lowe expects organ and tissue donation following post-mortems to



Carol Wainwright leaves Alder Hey with her son Joseph and the remains of his twin, Oliver.

drop still further. Similar declines followed recent protests about organ retention in France and Scandinavia, he notes.

In an effort to rebuild public confidence, the government is promising reforms and a stricter enforcement of existing laws. The laws allow tissues or organs to be retained after a post-mortem if relatives do not object.

Some UK pathologists say that threats of legal action are creating uncertainty even where the research uses tissue that would otherwise be destroyed, such as that removed during surgical procedures or for diagnosis.

"People have changed their attitude to tissue collection overnight," says Phil Quirke, head of histopathology at the Leeds Teaching Hospitals NHS Trust. "We are very uncertain about what we can and cannot do, so we've stopped collecting some material." His group's research into colon cancer will be "severely held up until the situation is clarified".

But president of the Pathological Society, Nicholas Wright, of St Bartholomew's Hospital, London, says Quirke is over-reacting. "I don't think there is any problem at all with using tissue excised during operations," Wright says.

Other researchers are concerned about possible restrictions on access to archived tissue, most of which was collected under older codes of ethical conduct. ■

♦ <http://www.rlcinqury.org.uk>

♦ <http://www.mrc.ac.uk/tissues.html>

Chinese scientific leaders denounce Falun Gong sect

Tokyo The Chinese Academy of Sciences (CAS) has denounced the Falun Gong sect, after five people claiming to be members set fire to themselves on the eve of the Chinese New Year. One of the five died and the others are in hospital.

Members of the Falun Gong have been protesting since the Buddhist group, which follows the teachings of US-based Li Hongzhi, was outlawed in China 18 months ago. Leaders have denied that the five were members.

At a forum held last week by the CAS, China's premier research organization, the academy's leadership took turns at criticizing the group, which practises meditation and exercise and claims 100 million followers worldwide. Those who see the ban as repressive and a violation of human rights do not understand the situation in China, said academy member Zhi-hong Xu, who is president of Beijing University.

UK government looking at plans to save Daresbury

London The British government is considering a proposal from physicists for a major new

multipurpose facility whose construction would secure the future of the Daresbury laboratory in northwest England.

Plans for the new £148 million (US\$220 million) Centre for Accelerator Science, Imaging and Medicine include a proton cyclotron for use in various research disciplines and in cancer treatment, an ion-beam facility for nuclear physics, a free-electron laser, and X-ray imaging equipment. A decision on the proposal is due to be announced later this month.

Daresbury is home to the United Kingdom's existing synchrotron radiation source, but its future has been in doubt since the government's decision to build a new £550 million synchrotron in Oxfordshire (see *Nature* 404, 323; 2000).

► <http://hep.ph.liv.ac.uk/~green/casim>

Charity grant vanishes in a puff of smoke

London The Cancer Research Campaign (CRC), one of Britain's leading cancer charities, has withdrawn a pledge to help build a new research institute at Nottingham University because the university is accepting money from British American Tobacco (BAT) for a separate project.

"The campaign had intended to launch a £2 million [US\$3 million] appeal to support

this lab, but in light of the university's decision, we have decided not to go ahead," says CRC director general Gordon McVie. The decision followed a ballot of CRC supporters in the region.

The university, which will use £3.8 million from BAT to establish a Centre for Corporate Social Responsibility, says it is "saddened" by the decision and that the new drug-discovery research institute is now in doubt. It says it acted within guidelines agreed by the CRC in accepting tobacco money.

Engineer takes the helm at Jet Propulsion Laboratory

San Diego NASA has named electrical engineer Charles Elachi as the new director of the



Charles Elachi:
new head of
NASA's JPL.

Jet Propulsion Laboratory in Pasadena, California.

Elachi has been a researcher and manager there for 30 years.

He was selected after an eight-month search to replace Ed Stone, who is retiring. Lebanese-born Elachi received his undergraduate degrees in France, and earned his doctorate at the California Institute of Technology,

which manages JPL for NASA. He is best known for work with radar sensing devices.

Global warming 'could cost \$300 billion'

Washington The potential cost of global warming is put at more than \$300 billion in a report from the United Nations Environment Programme (UNEP) and Munich Re, one of the world's largest re-insurance companies.

More frequent natural disasters, agricultural damage due to rising sea levels and subsequent stress on health care and water management could cost countries several percentage points of their gross domestic product by 2050, the report says.

The report was released just before 100 environmental ministers gathered in Nairobi for a meeting of UNEP's governing council.

No traces of modified DNA in poultry fed on GM corn

Tokyo Researchers at Japan's Scientific Feed Association say there is no sign of modified DNA or protein in chickens that had been continuously fed Starlink genetically modified corn.

Starlink is feared to cause allergic reactions in humans and has not been approved for human consumption, but it

found its way into the food chain last year (see *Nature* 408, 126; 2000), prompting the Japanese government to carry out fresh safety testing on its suitability as food for humans and animals.

The tests found no abnormalities in 256 chickens and no modified DNA or protein in blood, liver and muscle samples. The results suggest that eating Starlink-fed chicken would not transfer modified DNA or protein to human beings. But Starlink is still not expected to be approved for human consumption in Japan.

New Canadian body hit by funding fears

Montreal Researchers have expressed anxiety that the new Canadian Institutes of Health Research (CIHR) may fail to meet expectations because it lacks a solid financial basis for long-term planning.

Just days after the CIHR made public its first round of grants (see *Nature* 409, 549; 2001), a lobby group for the health research community issued a statement demanding details of how the new agency will be funded.

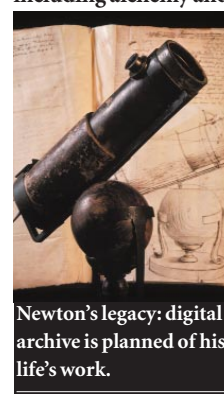
The Coalition for Biomedical and Health Research said that the CIHR was already rejecting "a majority of first-rate, scientifically valid proposals due to lack of funds" and called on the government to

commit to funding for the agency beyond April 2002, when current funding expires.

Major grant to put Newton's archive on the web

London Supported by one of the largest grants ever awarded by the British Academy—£330,000—a group of science historians is launching a 15-year project to complete an electronic edition of Sir Isaac Newton's unpublished manuscript archive.

Newton's intellectual ambitions stretched beyond his achievements in mathematical physics, which transformed science in the seventeenth century. Somewhat covertly, they embraced less scientifically rigorous projects including alchemy and theology.



Newton's legacy: digital archive is planned of his life's work.

Some 40 scholars will now digitize these documents, along with Newton's better known works. The project will be based at Imperial College, London, and all the material will be freely available on the web.

♦ www.newtonproject.ic.ac.uk

CORBIS

Testing times for BSE

No one knows whether the diagnostic tests being used to search for BSE infection in Europe's cattle can reliably detect animals incubating the disease. Given this limitation, asks Quirin Schiermeier, what is the testing programme likely to achieve?

The spectre of bovine spongiform encephalopathy (BSE) is stalking continental Europe. France last year recorded 138 cases, and countries that had judged themselves BSE-free have been shocked out of their complacency. In Germany, the country's first home-grown BSE cases have led to two ministerial resignations; only last month, beef sales in Italy collapsed as the country's first case came to light.

Given widespread exports of potentially infective British animal feed¹, the fact that BSE is now a pan-European problem should be no surprise. But for politicians who have been in denial, there is a new urgency to establish the scale of the epidemic. To find some answers, the European Union (EU) last December agreed to a programme of diagnostic testing of slaughtered cattle. Although the tests should put some figures on the extent of Europe's BSE problem, consumers anxious to avoid any risk of infection cannot rely on the testing regime. The tests being used perform well in identifying cows showing symptoms of BSE, but it is so far unclear whether they can reliably identify animals incubating the disease but showing no symptoms.

This has already caused controversy. On 6 December, Britain's *Independent* newspaper lambasted the European Commission for its reliance on a test that "has never been properly validated". Commission officials reacted angrily, arguing that the tests had only ever been presented as an additional safety measure. "The EU is perfectly aware of the deficiencies of the tests," responded Andrea Dahmen, spokeswoman for research commissioner Philippe Busquin. "The tests are a very useful additional measure to restore consumer confidence. However, the most vital thing is that our safety legislation is respected, especially the removal of risk materials from slaughtered ruminants."

The public was left wondering what to believe: would the tests protect them from this terrible disease? And if not, why should consumer confidence be restored? For now, those questions remain unanswerable. But despite these limitations, the EU's testing programme should allow epidemiologists to



Europe under a cloud: as the BSE crisis escalates, German farmers protest about the planned culls of their herds (left) and a researcher in Spain tests a carcass for signs of the disease.



begin to assess the extent of BSE in mainland Europe. Until now, the only real certainty is that there has been widespread under-reporting of the disease.

Accelerated timetable

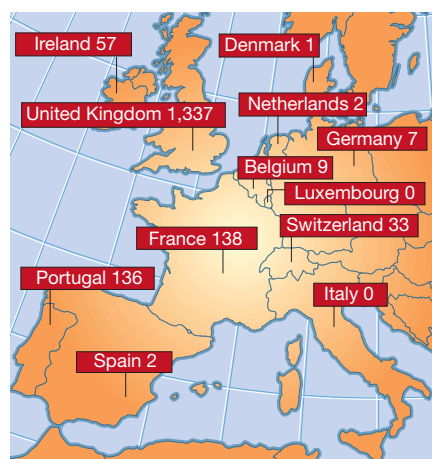
The testing programme has two phases. From January this year, cattle that have shown suspicious symptoms must be tested with one of three approved diagnostic tests. From July, this requirement will be extended to all cows coming to slaughter aged 30 months or more. Several EU states have accelerated this timetable — France and Germany, for instance, have been testing all 'over 30-month', or OTM, cattle since

January; and Germany has in recent weeks extended its testing to all slaughtered cattle older than 24 months.

The OTM rule has an empirical basis. Although there have been some 50 reported cases of BSE in animals younger than 30 months, 99.97% of the roughly 180,000 BSE cases recorded in Europe since 1986 — the vast majority of them in Britain — have fallen into this age group. In Britain, consumption of OTM cattle is banned.

The tests approved by the EU have subtle differences, but all use antibodies to detect the 'prions' that accumulate in the brains of cattle with BSE. These rogue proteins are thought to cause the disease by converting a protein called PrP into their own, misshapen form — known as PrP^{sc} or PrP^{res}. In 1999, Jim Moynagh of the European Commission's Consumer Policy and Health Protection Directorate General and Heinz Schimmel of the Institute for Reference Materials and Measurements in Geel, Belgium, subjected four diagnostic tests to validation studies. Each was put through its paces on 300 samples of brain tissue from cows with BSE and 1,000 samples from healthy New Zealand cows. Three of the tests — made by Prionics of Zurich, Enfer Scientific in Tipperary, Ireland, and the French Atomic Energy Commission (CEA) — emerged with a 100% record, recording neither false negatives nor false positives². In practice, the tests do have a very small error rate when tested on tens of thousands of samples.

But the problem is that this validation did not assess the tests' ability to detect infection



Crossing the borders: confirmed cases of BSE throughout Europe during 2000.

in cows incubating BSE — which are, after all, those that the EU's testing programme hopes to identify. So far, the best way to detect pre-clinical BSE infection has been to take a sample of brain tissue from the cow in question and inject it into the brains of mice; if these animals later develop the disease, then the cow was incubating BSE.

Because this bioassay takes many months to run, it is impractical as a working diagnostic tool. But new evidence suggests that at least one of the approved tests is similarly sensitive. The CEA test, produced commercially by Bio-Rad of Hercules, California, was recently tested against the mouse bioassay in its ability to detect PrP^{Sc} in diluted samples of brain from cattle with BSE. The results, published last month³, revealed that Bio-Rad's test and the mouse bioassay performed equally well, even on samples diluted to one in 1,000. "The results were at least as accurate as those provided by the mouse bioassay," says Moynagh.

"This looks like a very good test," agrees Paul Brown, a prion disease researcher at the US National Institute of Neurological Disorders and Stroke in Bethesda, Maryland. Nonetheless, its ability to perform well with diluted samples from BSE cases does not prove that the test will reliably identify pre-clinical BSE. "The whole testing programme is a waste of time if tests cannot detect pre-clinical disease well enough," Brown says.

Deliberately infected

Providing the evidence that Brown wants to see will require the tests to be validated on tissues from cattle that have been deliberately infected. Only then will it be possible to determine whether any test can provide a pre-clinical diagnosis; and, if so, to determine the point during the course of infection at which diagnosis becomes reliable.

Just one laboratory can provide the necessary samples. In 1998, at Britain's Central Veterinary Laboratory in Weybridge, Surrey, 200 calves were infected with BSE at two different doses. At three-month intervals, groups of four of them, plus two healthy controls, are slaughtered and samples of their fluids and tissues are harvested and frozen.

This followed from an earlier study, in which 30 cattle were infected with BSE and then slaughtered at regular intervals to investigate, using the mouse bioassay, which tissues might pose a risk to human health, and to determine when they become infective⁴. Samples from the first study have all been used up. But Gerald Wells, the veterinary pathologist in charge of both studies, says that scientists who want access to frozen samples from the ongoing study to validate diagnostic tests are welcome to make requests. "If people want pre-clinical tissue," he says, "then we can make it available."

If the tests currently in use can reliably detect pre-clinical BSE, then the EU's testing programme should prove invaluable to epi-

demiologists striving to analyse the dynamics of the epidemic.

Using incomplete data, epidemiologists have already made some progress. Based on the available French data, for instance, Christl Donnelly of London's Imperial College last year estimated that since 1987 at least 1,200 French cattle have been infected with BSE⁵. Similar analyses are possible for countries such as Switzerland, which has instituted its own programme of BSE testing. But for many European countries, epidemiologists cannot begin to bring their skills to bear. "Estimations are practically impossible if there are only a few reported cases," says Donnelly.

The current generation of diagnostic tests may prove to be of limited value in detecting pre-clinical cases. In any case, it would be much better to have a simple blood test that could be used on live animals, rather than one that requires samples of brain tissue. In that regard, some experts are encouraged by the discovery last year that prions bind to a blood protein called plasminogen⁶ — an interaction that might be used to develop a blood test. Others point to the work of Mary Jo Schmerr

of the National Animal Disease Center in Ames, Iowa, who has shown that it is possible to detect the tiny quantities of prions in the blood of sheep infected with scrapie, a related disease⁷. In this test, the prions compete with fluorescently labelled synthetic PrP proteins to bind to antibodies, and the resulting mixture is separated and analysed by being passed through a capillary.

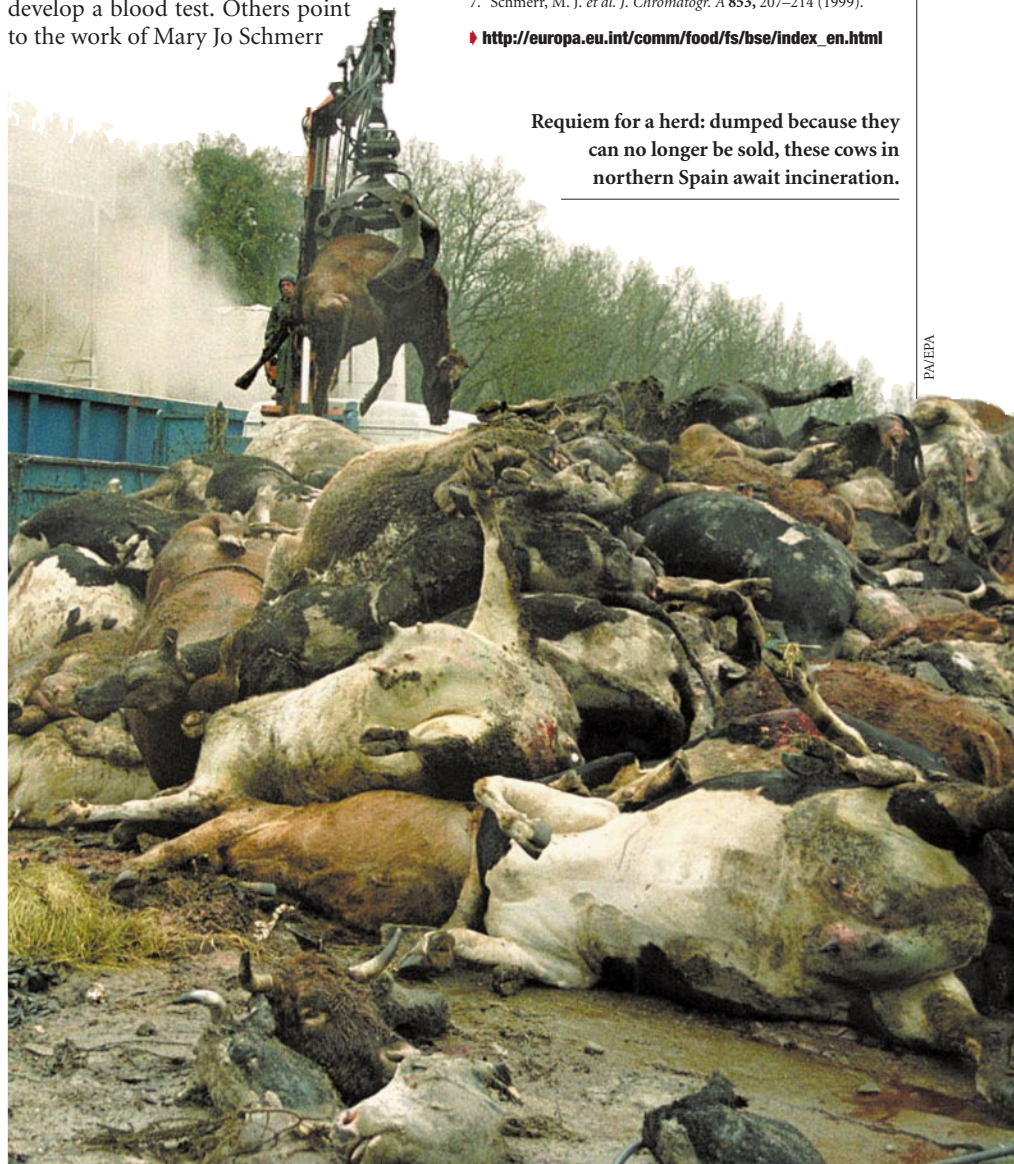
Whether or not the answers can come from current diagnostic tests, experts agree that thorough testing of its cattle herds offers the best route for Europe to get to grips with its BSE problem. "We want to understand how the epidemic behaves," says Adriano Aguzzi, a neuropathologist at the University of Zurich. "Since we estimate a massive number of unreported cases in many countries, more testing is the only way to find out."

Quirin Schiermeier is *Nature's* German correspondent.

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♦ http://europa.eu.int/comm/food/fs/bse/index_en.html

Requiem for a herd: dumped because they can no longer be sold, these cows in northern Spain await incineration.



PAVEPA

In search of a cure for CJD

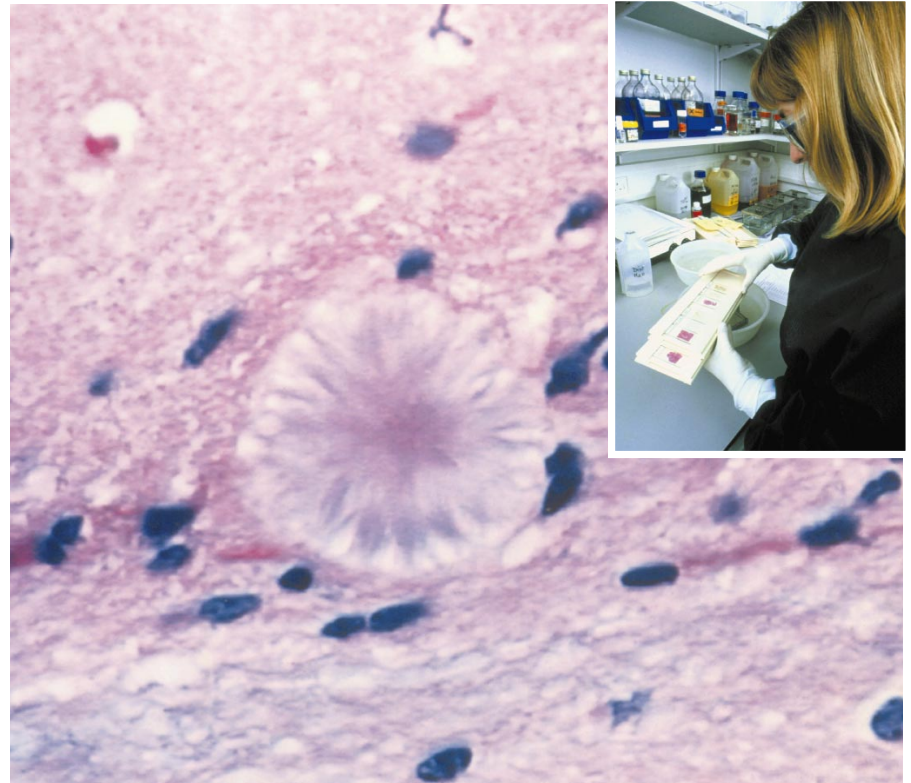
Many thousands of people may be incubating the human form of BSE. Could drugs or other therapeutic agents prevent them from succumbing? Clare Thompson reports on the race against time to find a treatment.

No one knows how many more people will join the 92 individuals who have so far fallen victim to the human form of bovine spongiform encephalopathy (BSE). With luck, there may never be more than a few hundred deaths from variant Creutzfeldt–Jakob disease (vCJD), as human BSE is known. But experts cannot rule out the awful possibility of an epidemic affecting many thousands of people — which is why finding a way to prevent the deadly neurological damage wrought by the rogue proteins, or prions, thought to cause these diseases is a top priority.

As with efforts to establish the scale and distribution of the BSE epidemic in Europe's cattle (see pages 658–659), much depends on the ability of scientists to devise diagnostic tests that can spot infection before any symptoms appear. By the time the symptoms of vCJD have become apparent, the patient's brain is probably irretrievably damaged. "It is far too late," says Moira Bruce, a prion disease researcher at the Institute for Animal Health's Neuropathogenesis Unit in Edinburgh.

But if a reliable diagnostic test can be found, research from the labs of leading prion researchers suggests that it might just be possible to save infected people from the nightmare of vCJD. Whether therapies can be developed on the timescale required, however, remains to be seen. And even if they can, public subsidies will probably be needed to bring them to market.

The first signs that drugs might delay the onset of prion diseases came in the mid-1980s, from work on scrapie, a prion disease of sheep. In those days, BSE was unheard of,



and CJD was a neurological curiosity — the sporadic form of the disease, unrelated to BSE, arises in about one in a million people. Indeed, the very idea that these diseases might be caused by a misshapen protein converting a normal brain protein, called PrP, into its own, twisted form was then outside the scientific mainstream — most researchers believed that a virus was involved.

Slow-motion scrapie

In 1984, Heino Diring and Bernhard Ehlers of the Robert Koch Institute in Berlin showed that an antiviral drug called dextran sulphate could prolong scrapie's incubation period in mice infected with the disease¹. Dextran sulphate belongs to a class of compounds known as polyanions, and other members of this group seem to have stronger effects — Bruce and her colleagues in Edinburgh, for instance, are working with a less toxic compound called pentosan polysulphate, normally used to treat cystitis².

Following Diring and Ehlers's original discovery, a few other compounds have been found that also delay the onset of scrapie symptoms in rodent models of the disease. In 1989, researchers led by Maurizio Pocchiari of the Italian National Institute of Health in Rome showed that an antifungal drug called amphotericin B pulls off the same trick as dextran sulphate³ — and again, prion researchers are now working with more effective derivatives⁴.

Since then, the list of candidate drugs has continued to grow^{5–9}. And with drug companies starting to screen their huge libraries of

Irreversible damage? The characteristic plaques formed in brain tissue as a result of vCJD cause neurodegeneration and death.

compounds for potential CJD drugs, more should be on the way. John Collinge of Imperial College, London, for instance, is now collaborating with GlaxoSmithKline. "We are working on a high-throughput approach, looking at about 200,000 compounds to see which ones can stabilize PrP," he says.

Into the unknown

The problem is that for most of the candidate drugs, there is no clear idea of why they delay the onset of symptoms. Many of them are presumed to work by interfering with the conversion of normal PrP to the rogue, prion form, known as PrP^{Sc}. But without a detailed understanding of how the compounds work, it is difficult to design



Prion victim: Zoe Jeffries is the youngest person so far to succumb to variant CJD. She died last October at the age of 14.

We need human trials. Government may have to work hand in hand with a pharmaceutical company.

improved derivatives that could be developed into effective drugs.

This is why some experts believe that a more targeted approach may be necessary. One of the most exciting recent discoveries in this area comes from the group led by Claudio Soto of the Sero Pharmaceutical Research Institute in Geneva. Prions differ from normal PrP in that parts of the protein have been converted from one structural conformation, known as an α -helix, to another, called a β -sheet. "Several years ago, evidence surfaced that if a protein doesn't have a proline amino acid, then it is more likely to adopt a β -sheet formation," says Soto. "This prompted us to use proline to keep the structure of the α -helix intact."

Soto and his colleagues devised a sequence of 13 amino acids, called iPrP13, that contains three prolines, but otherwise closely matches the sequence of the part of PrP that gets converted into a β -sheet. Last year, the researchers showed that, *in vitro*, this amino acid converts PrP^{Sc} back to the normal, α -helix form¹⁰. It also substantially delayed the onset of symptoms in mice infected with scrapie.

Soto has since been trying to produce smaller peptides with a similar effect, as these are less likely to be broken down in the body — and has developed one that consists of just four amino acids. "The original peptide was a proof of concept," says Soto. "We now have to turn it into a drug." That will require further effort to prevent the peptide being degraded, and working out how to deliver it at high enough doses into the brain. But Soto is optimistic of making good progress. "We plan in the next two years to move into human clinical trials," he says.

At the University of California at San Francisco, the father of prion theory, Stanley Prusiner, is working with protein chemist Fred Cohen on another targeted strategy. Prusiner and Cohen maintain that an undiscovered protein, dubbed 'protein X', helps to

convert PrP into PrP^{Sc}. They have identified a region within PrP that appears to interact with protein X (ref. 11). Focusing on compounds with structures that can bind to this region, the San Francisco team has screened for those that inhibit the conversion of PrP to PrP^{Sc} — eventually finding two that did the job and also had low toxicity¹². But Cohen admits that they are some way from having a viable drug. "The activity would have had to be 100-fold more potent," he says.

Prion patrol

Other researchers wonder whether it might be possible to prevent the deadly prions from getting into the brain in the first place. Before they enter the central nervous system, prions multiply in immune cells known as follicular dendritic cells. These cells can be temporarily inactivated using a soluble form of a protein called lymphotoxin- β receptor. And last year, two teams, one led by Bruce, the other by Charles Weissmann of Imperial College and Adriano Aguzzi of the University of Zurich, showed that this treatment could delay the onset of scrapie in mice^{13,14}.

Perhaps the most intriguing possibility has been borrowed from Alzheimer's disease research, where a vaccine to generate antibodies against a protein called amyloid- β is showing good promise as a treatment^{15,16}. Collinge and Cohen are among the researchers now experimenting with antibodies against PrP^{Sc}, to see if these can work a similar magic.

But whichever strategy proves most effective, one important obstacle will remain — money. Preventing thousands of cases of vCJD is a noble goal, but the harsh realities of the drug industry demand a bigger market, if companies are to meet the huge costs of developing a therapeutic agent. "We need more evaluation and human trials," says Collinge. "And who would pay for that remains an important question. It may get to the stage where government would have to work hand in

Degenerating: an MRI scan of a human brain with vCJD.

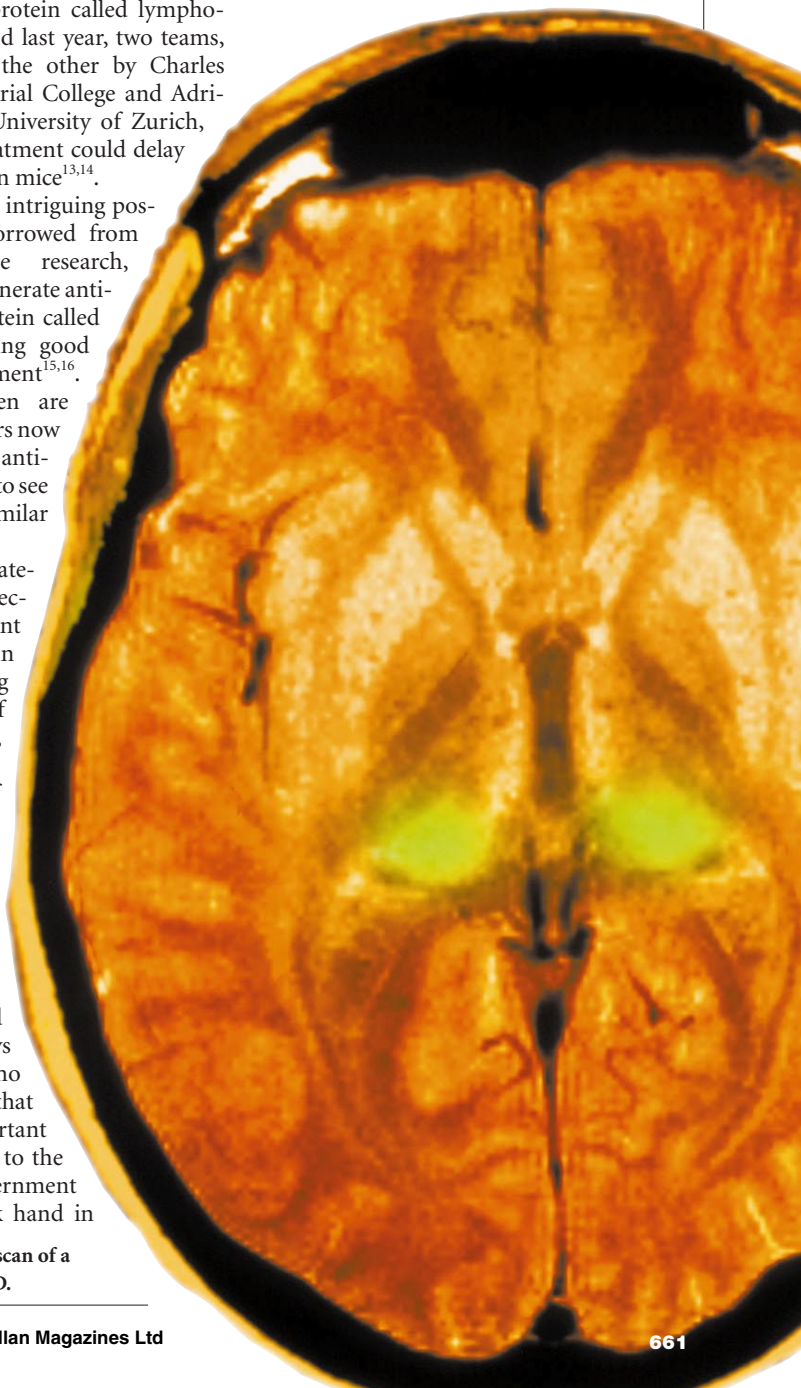
hand with a pharmaceutical company." ■

Clare Thompson is a medical writer based in London.

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John Collinge: hopes antibodies may defeat prions.



SIMON FRASER/SPi

Leaders need to realize that science can offer a route out of poverty

Sir—Recently, *Nature* has given a lot of attention to science and technology in Latin America^{1–4}. A key issue, however, needs to be addressed: in Latin America neither the public nor the private sectors, nor governments, acknowledge that science and technology development is an essential prerequisite for the cultural and economical advancement of society.

Scientific research is viewed as a marginal activity in Latin America. The construction of a strong local system of science and technology is not considered an important route towards development and cultural literacy in countries where most people lack basic health services, education and social security. It is not considered important for people's welfare amid poverty, underemployment, social marginalization, corruption and violence.

This attitude translates into fragile educational systems at all levels: poor academic standards among teachers, overcrowded classrooms, nonexistent or inadequate audiovisual equipment, violence at school, and a shortage of computers, laboratories and libraries.

Our countries allocate meagre budgets for scientific research and technology R&D, both in absolute value and in terms of the gross national product (GNP). This results in very low publication rates in local academic outlets (if any exist) and international peer-reviewed journals. This in turn, reduces the number of people participating in these activities and leads to frustration among enthusiastic young students, who lack any motivation to dedicate their lives to such endeavours. This causes an excessive brain-drain to developed countries, reinforcing the downward spiral: lack of opportunities to reverse underdevelopment, increasing poverty, and the ever-widening gap from developed countries in all aspects.

Historically, Colombia's investment in science and technology has been one of the lowest in Latin America, amounting to much less than 0.25% of the GNP. In 1993–94 a thorough study was conducted to diagnose and make recommendations concerning the state of education, science and technology in Colombia⁵. The study was carried out by a team that included, among others, such eminent members as biochemist Manuel Patarroyo, neuroscientist Rodolfo Llinás and microbiologist Angela Restrepo, and the Nobel prize-winning writer Gabriel García Márquez. Among the recommendations was an increase in science and technology

education and research expenditure to 2% of the GNP by 2000.

Last year, the entire budget of Colombia's Institute for Science and Technology (Colciencias) amounted to less than US\$15 million, around 0.17% of the GNP. The severity of this crisis was dramatically illustrated last month, when a first-world-based private bank repossessed all the scientific equipment of Patarroyo's Instituto de Inmunología, "on the basis of outstanding debts"⁶. During recent years, this situation in Colombia has been exacerbated by a deep economic recession coupled with a bloody civil war.

In addition to the grossly insufficient development of science and technology in Latin America (which, I believe, is true of the developing world in general), the international scientific community imposes its own burdens. Journal subscriptions, memberships to scientific societies, page charges for accepted papers, registrations at scientific meetings, are all assigned with the research budgets of wealthier countries in mind. Hence they contribute to preventing scientists in low-income countries from participating in the worldwide scientific community.

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Secret behind Hungary's stellar intellects

Sir—Vaclav Smil's charming essay (*Nature* 409, 21; 2000) revealed the *genius loci* of turn-of-the-century Budapest. As he says, some of the greatest figures in twentieth-century science came from one small quarter of the city: Leo Szilard, Dennis Gabor, Eugene Wigner, John von Neumann and Edward Teller were born there (along with the writer Arthur Koestler) between 1898 and 1908, shortly after Theodore von Kármán and George de Hevesy. Andrew Grove and John Kemeny were later born in the same area.

But Smil omitted mention of Szilard's brilliant explanation of how this had come about. When Enrico Fermi, wondering at the immensity of the Universe and the certainty of life on planets orbiting stars in our Galaxy, asked why no beings from outer space had arrived, Szilard responded: "They are among us, but they call themselves Hungarians." How could one

be sure? "Because they speak a language no one else can understand!"

I refer readers to *The Leo Szilard Centenary Volume*, edited by George Marx and published by the Eotvos Physical Society in 1998 (Fo Utca 68, Budapest H-1027, Hungary), for scientific documentation.

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Why didn't flood of print launch science in China?

Sir—Adrian Johns wrote in his Words essay¹ that "science originated partly from a need to master as many [words] as possible", and that the impetus for this need came from "the development of printing in the mid-fifteenth century".

Printing was, of course, first developed in Tang Dynasty China (AD 618–907). The earliest surviving example of printed text is a 'dharani sutra' scroll printed between 704 and 751, now kept in Pulguksa Temple, Kyongju, Korea, and the earliest extant example of a printed book is the *Diamond Sutra* of 868, now kept in the British Museum. Both were printed in China.

It is therefore doubtful that the sudden deluge of printed material in fifteenth-century Europe was a major cause of the Scientific Revolution. It has been estimated that, by 1700 or even 1800, more written and printed pages existed in Chinese than in all other languages of the world put together^{2,3}. Creel also estimates that, until the middle of the eighteenth century, more books had been published in Chinese than in all other languages put together⁴.

The most prolific printing period in history was probably not the European Renaissance, but the Qing Dynasty (1644–1911). It was noted that "of the quarter of a million titles of Chinese publications known to have accumulated throughout the dynasties, no less than one half were produced during this period, the greatest amount in all history"⁵.

If the ready availability of printed material were indeed the main impetus for the Scientific Revolution, it would have happened in China, and not in Europe.

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Fit for the seven-league boots

The story of a man who sought to harness nature for the American good.

A River Running West: The Life of John Wesley Powell

by Donald Worster

Oxford University Press: 2001. 646 pp. \$35

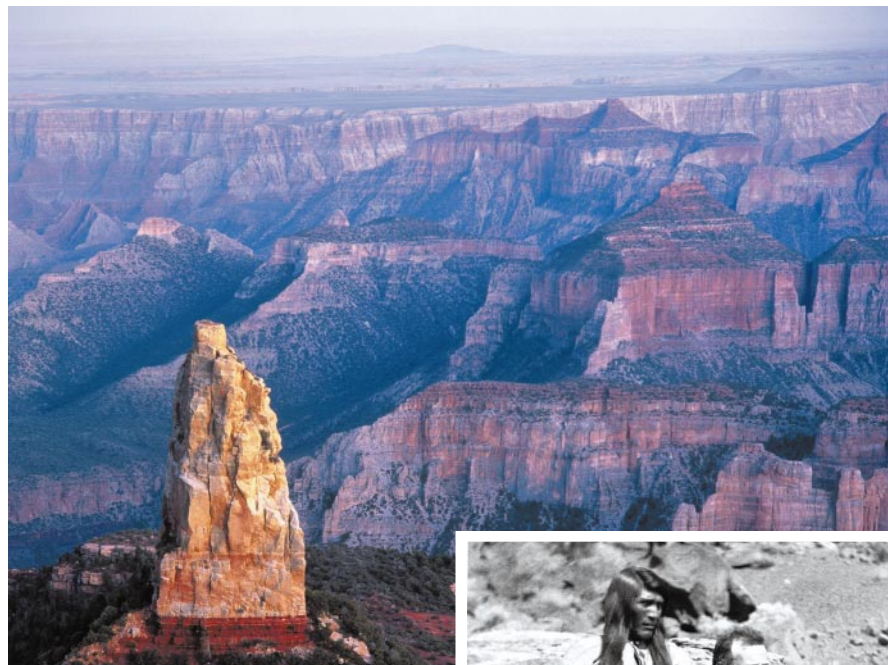
Richard White

As oxymoronic as the phrase may sound, the late nineteenth century was an age of heroic bureaucrats, and John Wesley Powell, the most heroic of the bureaucrats, was also a man of science. He became simultaneously head of the US Geological Survey and the Bureau of American Ethnology, which were then two cornerstones of government science. But before that he had been a wounded and mangled veteran of the Civil War and the one-armed leader of the first expedition intentionally to run the Colorado river. Donald Worster has written a compelling and powerful biography of Powell that tells us much about the American West, government science and the ways in which science was linked with the wider world.

At 31, Powell had done little, in Worster's nice phrase, other than "acquire a character". His character contained the tensions — moral and emotional — that arose from his Methodist upbringing. It was also shaped by his nationalism, a passion for science, belief in progress, physical courage, a sense of duty and substantial personal ambition. Powell's church was the American nation, his creed was democracy and science, and he would have envisaged salvation — for all his personal ambition — as collective rather than individual. His character would be felt in two places: the American West and Washington DC.

In his famous Colorado river expedition, Powell led a group of relatively disciplined amateurs on a publicly funded voyage of scientific discovery. Writes Worster, he wanted "to possess the vast interior space of the Colorado River as his own intellectual property, to measure it off and stake it out in seven-league boots". What that meant was not only mapping and discovery, but also making natural-history collections and studying the geology.

Worster gives a wonderful account of the expedition, in his estimation one of the great American explorations. It became Powell's popular and intellectual capital, and he invested it wisely. The expedition was extended into an ongoing survey and became the basis of Powell's own government survey of an American West already, in the 1870s, thick with government surveys. All were at work mapping the West and cataloguing its resources, the national interest in many cases proving to be remarkably congruent with the

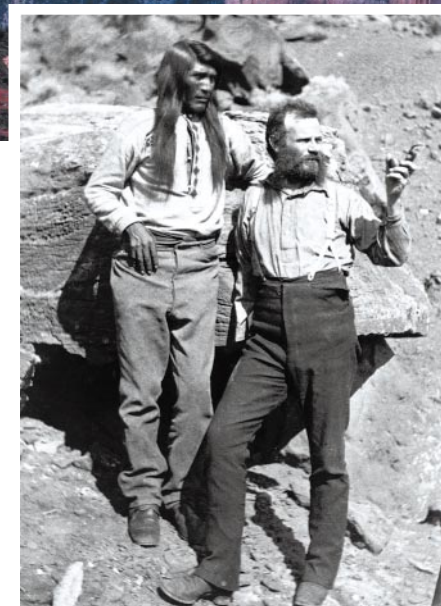


Grand designs: if Indians were one pole of Powell's efforts, the Grand Canyon was the other.

interests of the railroads and mining companies. Powell's survey marked itself off from the others by its interest in Indians and its concentration on the Colorado Plateau that stretched from the southern end of the Great Basin into Arizona. Worster emphasizes Powell's interest in the Indian peoples, his sympathy for them, and his relentless desire to change them in the pursuit of his own progressive nationalism.

If Indians were one pole of Powell's efforts, the Grand Canyon became the other. Powell's artists, geologists and photographers played a key role in the story of how, in environmental historian Stephen Pyne's phrase, the Grand Canyon became grand.

As a scientist, Powell was quick and intuitive. He sketched out large patterns and hypotheses, but he was impatient with the careful work needed to follow them up. His crucial importance lay in his ability as an organizer and recruiter of scientists, and as a negotiator with Congress. He detached the two government agencies he eventually led from the constituencies that would seemingly capture them: the US Geological Survey became far more than the tool of the mining industry. Instead, Powell focused on palaeontology, topography, studies of the Ice Age and the geology of places that had no hope of providing mineral resources. He did not allow applied science to dominate government science. The numerous publications of Powell's survey and later his agencies



served not only his own interest but also a larger ideological interest in educating the American people.

Powell was also more than willing, indeed eager, to put science to work in shaping public policy. As a reformer and social theorist, he thought in terms of progress from savagery, to barbarism, to civilization. He saw history as the gradual triumph of science and scientific thinking. Indians, who represented the savages in his scheme of thinking, were only "whites without science". Powell believed that, as science extended its domains ever farther into the realms of ethics, then egotism and selfishness would give way to altruism. The laws of natural evolution were not the laws of social evolution, and, for Powell, "the war for survival must give way to a war on nature". There was a millennial aspect to all of this that Powell's Christian forebears would

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book reviews

have recognized, but science had replaced faith as the redeeming agent. Science would direct the American people "in a legitimate course of development".

The most famous aspect of Powell's education of the American people was his attempt to make American settlement conform to what he regarded as the logic of the arid West, where scarce water limited both the extent and form of settlement. There were really two attempts at this — his "Report on the Arid Lands of the United States" in 1878, and what began as a partnership with Senator William Stewart of Nevada to irrigate the West in 1890. Both failed, and their failure forms the central story of Worster's book.

Worster has two standards for measuring Powell the reformer: respect for nature and the struggle for social justice. Powell's record is mixed on both, and the gap between Powell's actions and Worster's standards creates a productive tension in the book. But this is not a case of an author imposing modern standards on the past; rather, nineteenth-century concerns resonate with modern concerns in an interesting and revealing way. Powell's "Report on the Arid Lands of the United States" was, according to Worster, trapped in fundamental contradictions. It identified a natural pattern that Powell wanted to use as the basis for a social pattern. "Nature in the West had drawn the lines that law, politics, land and water use, and economic development must obey." Powell paid tribute to nature only in order to transform it. The survey was a search for the best site for dams that would plug every rivulet in the West and allow no drop to go to waste. It served a populist agrarianism that has Worster's sympathies, but it was still an assault on nature. For Worster, who believes deeply that nature provides patterns that humans should follow, it was the Achilles heel of a flawed, important and truly revealing figure. ■

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Taxonomy and the blues

Nabokov's Blues: The Scientific Odyssey of a Literary Genius

by Kurt Johnson & Steve Coates
Zoland Books: 2000. 372 pp. £18.99, \$27

James Mallet

Vladimir Nabokov the towering man of letters was also the geeky and shambling lepidopterist who, every summer, roamed the uplands of North America or Europe with a butterfly net. *Lolita* (1958) and other novels were actually written while he was on butter-

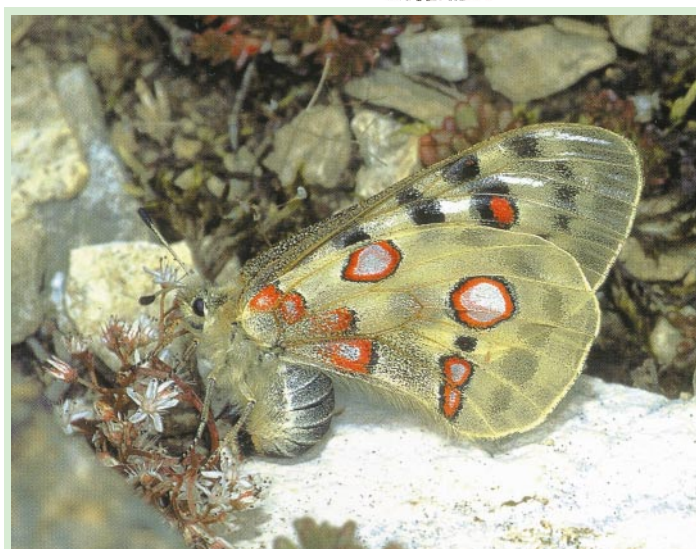
fly-collecting trips in the Rockies. Nabokov won international fame as a novelist and poet after *Lolita*, but he was even more proud that a handful of specialists had named butterflies after him. In spite of its subtitle, *The Scientific Odyssey of a Literary Genius, Nabokov's Blues* is not primarily another biographical work about the great writer. The many references to butterflies in Nabokov's novels and poems, and his taxonomic work on the 'blues' (Lycaenidae), are discussed at length, but the odyssey in the subtitle properly belongs to author Kurt Johnson, a New York financier and lepidopterist. The real hero of *Nabokov's Blues* is taxonomy; its supporting cast, in addition to Nabokov and Johnson, includes Hungarian PhD student Zsolt Bálint, Israeli engineer Dubi Benyamini and Peruvian entomologist Gerardo Lamas.

Nabokov's Blues thoroughly captures the joys and frustrations of taxonomic discovery. With Johnson and Steve Coates, a *New York Times* journalist, the reader battles through mudslides and guerrilla roadblocks to remote sites in the Andes and Caribbean, dissects butterfly genitalia under a microscope, visits isolated European museums in search of dusty and forgotten type specimens, peruses the literature, vindicates Nabokov's pioneering taxonomy, and finally races to describe and publish new discoveries ahead of competitors. This may be the first book to exploit

alpha-taxonomy for its thrill value. It is a most unlikely bestseller, but the book succeeds by romping enthusiastically around the arcane world of butterflies and butterfly taxonomists.

Most of us know about the extinction crisis in biological diversity. Less publicized is the fact that we can't even estimate the scale of this global disaster because of a crisis in taxonomy itself: we haven't a clue how many species there are, even to an order of magnitude (estimates vary between 1 and 100 million). I can download onto this very computer information on stars billions of light years away, find detailed information about every square metre of the Earth from among terabytes of online satellite data, and identify each of the 10^8 base pairs of DNA in the genome of the tiny fly buzzing round my glass of claret. But there is no universal taxonomic database. In spite of its fundamental importance in biology, taxonomy is today at one of its lowest ebbs, and only survives through the fevered activity of a few poorly paid but dedicated museum curators, as well as a Dad's Army of retirees, amateurs and volunteers. *Nabokov's Blues* provides a much-needed portrayal of this close-knit yet scattered international community.

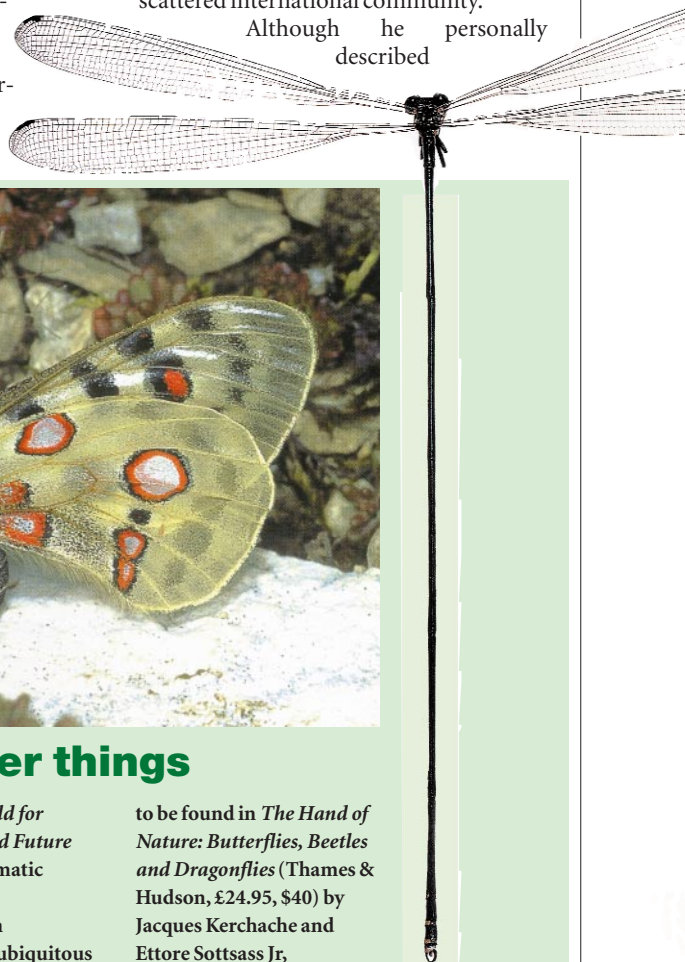
Although he personally described



Wings and other things

Phil Schappert, the author of *A World for Butterflies: Their Lives, Behavior and Future* (Firefly, \$35), calls them "the charismatic megafauna" of the insect world. His lavishly illustrated book provides an introduction to this ephemeral but ubiquitous arthropod, together with the reasons that many species have become endangered. Further intimate portraits of these and other insects are

to be found in *The Hand of Nature: Butterflies, Beetles and Dragonflies* (Thames & Hudson, £24.95, \$40) by Jacques Kerchache and Ettore Sottsass Jr, with photography by Patrick Gries. The book is a photographic record of Kerchache's insect collection.



many new species of 'hairstreak' butterflies (like Nabokov's blues, also Lycaenidae), Johnson himself is far from respected as a taxonomist. Even Johnson's unpaid associate status at the American Museum of Natural History was terminated in 1996. In an e-mail, Johnson himself now admits to "10–20% or less errors" in his earlier taxonomic work — although others put his synonymy rate higher (the criticisms do not apply to his recent collaboration with Zsolt Bálint). For a taxonomist, it seems extraordinarily careless that Johnson refers in *Nabokov's Blues* to species "epitaphs", when he means "epithets" (names). Such errors are unacceptable to taxonomists, who take pride in their accuracy and orthography. Nonetheless, in his new metamorphosis as bestselling author, Johnson keeps any bitterness he may feel towards critics out of this lively book — hints that he is a renegade only add to the charm. Instead, Johnson and Coates advertise a much more valuable and convincing message: taxonomy is fun!

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Crash, bang, wallop

Science of Percussion Instruments

by Thomas D. Rossing
World Scientific Publishing: 2000. 208 pp.
£16, \$25

Bernard Richardson

Have you noticed a strange new phenomenon at concert halls? Percussionists, who used to stand at the back of the orchestra tapping out the rhythm or punctuating the odd climax with a cymbal crash, are increasingly taking centre stage. Armed with a vast array of instruments, they treat the audience to virtuosic performances which seem to rely as much on athletic prowess as musicianship. If you have difficulty in putting a name to half of these instruments, or have ever wondered why and how they produce such a wide range of musical sounds, then this authoritative book by an expert will be a welcome addition to your bookshelves.

Percussion instruments are a little different from the other regular members of the orchestra. Strings, woodwind and brass combine some type of oscillator (string or reed) with a resonator (a wooden box or air column) in such a way as to produce sustained sounds with strictly harmonic overtones. This is why we perceive these instruments as having a very definite pitch and why their sounds can be combined so harmoniously. In contrast, percussion instruments



Beating all comers: there's more to drums than even the band Iron Maiden could have dreamt of.

combine the oscillator and resonator in one, producing sounds through the transient excitation of the natural vibrations of bars, plates, stretched membranes and shells made from a host of different materials. The ensuing vibrations of these structures tend to produce short-lived sounds comprising inharmonic overtones. It is this rather haphazard overtone structure, and the way it develops with time, which determines the sound quality of each instrument. The key to understanding the acoustics of percussion instruments is thus to study the modes of vibration of the different groups of instruments — and that is largely what this book is about.

Thomas D. Rossing has written this book for a general readership and therefore assumes no particular scientific background. Rossing is professor of physics at Northern Illinois University in DeKalb, and has been actively involved in musical acoustics for more than 30 years. Much of his own work has concentrated on the acoustics of percussion instruments, and he has also been active in promoting science through his work for the Acoustical Society of America and the American Association for the Advancement of Science (of which he is an ex-president).

In the book, so that they can be visited at any time, bits of physics or descriptions of specialized techniques (such as the holographic interferometry used to visualize vibrations) are presented in "interludes". Whether or not these interludes contain sufficient background material to explain essential concepts to musicians and instrument-makers is debatable. However, the

book is certainly accessible to scientists interested in music, although I would have liked to have seen a little more on psychoacoustics, the science of measuring or explaining how we hear. A section on materials and damping, and a more fundamental approach to sound radiation would also have been welcome. Like other books on musical acoustics, this one is organized logically with sections on each of the major families of instruments (making it useful as a reference text).

What singles this book out is the sheer diversity of instruments covered. There are gamelans and gongs, bongos and bodhráns, tam-tams, thunder sheets and typewriters — if it makes a noise when you hit it, you will probably find it in this book. There is inevitable overlap with material from one of Rossing's previous books, *The Physics of Musical Instruments* (co-authored by Neville Fletcher; Springer, 1991), but there is sufficient new scientific material and a wealth of interesting snippets about the origins, use and manufacture of instruments to warrant this new publication.

A good third of the book is, quite reasonably, devoted to bells and Caribbean steel drums. These are part of a special class of 'tuned' percussion that demonstrates how, in the hands of skilled instrument-makers, the modes of vibration can be brought into near-exact harmonic alignment, giving them a precise pitch. Bell tuning is an example of a lost art rediscovered in the late nineteenth century, partly as a result of the monumental acoustical work of Lord Rayleigh. Modern bell tuning is done electronically on a large vertical lathe, gradually

paring metal away at strategic points; depending on whether metal is removed predominantly from antinodal or nodal areas (vibrating or non-vibrating areas), modes can be tuned up or down in frequency. Recently, science has helped to develop new types of bells. Traditionally, bells have a strong minor-third overtone, which is what produces their characteristic doleful sound. Rossing describes how the combination of traditional know-how with modern finite-element methods has created a major-tuned carillon bell, now available commercially.

Rossing's account of the complex metal-lurgy and manipulations involved in steel-drum construction makes for similarly fascinating reading, especially when you consider that many pans are still constructed from used oil drums over an open fire in back-street workshops! The universal appeal of these steel drums is now creating such a demand that manufacturers are having to seek new materials and methods of construction. The sort of research and level of detail covered by this book should surely help in this goal.

In short, this is a very welcome book. It is fair to say that the science of percussion instruments would not have advanced anywhere near so far without the tireless enthusiasm and passion of Rossing and his students. Committing this vast experience to text goes a long way towards elevating the status of percussion instruments alongside those of the other instruments of the orchestra.

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Science in culture

The harmonious hand

Marin Mersenne and the science of memorized music.

Martin Kemp

The history of the study of the human hand is permeated by a sense of awe. Described by Aristotle as the "instrument of instruments", the hand was seen as the unique tool of the intellect and as the bodily organ that best denoted the distinction of humans from "brutes". Not only was it wondrously engineered, but it also served as a subtle register of emotion, as a device for computation, as a formal means of communication in rhetoric and sign language, and as a prime visual site for the exercise of the 'art of memory'. In this last capacity, it performed a particularly notable role in music.

The eleventh-century theorist and teacher Guido d'Arezzo, who was responsible for important innovations in musical notation and sight-singing, is also credited with the invention of a much-used system that assigned musical pitches to the joints of the hand. In the words of the great seventeenth-century French composer Jean-Philippe Rameau: "It will be seen readily enough that the five fingers of the hand are very capable of representing the five lines upon which music is written: for if one contemplates or imagines the hand held well open, with the little finger nearest the ground, one may see the five lines with their spaces — which are gaps separating the lines formed by the fingers."

In an age where memorization of complicated sets and sequences was crucial, the Guidonian hand served as a significant aid in the learning of intricate scores, particularly by young singers. The many illustrations in manuscripts and printed books generally display the left hand, leaving the right index finger available for pointing by the instructor.

No one made more ambitious use of the hand

as a site for musical notation than Marin Mersenne (1588–1648), Christian philosopher, mathematician, encyclopaedist of music, pioneer of acoustics and the man personally responsible for a pan-European 'internet' of correspondence on all manner of intellectual concerns. Among other claims to fame, Mersenne seems to have been responsible for the addition of the note *si* at the end of the sequence *ut* (later *do*) *re mi fa sol la*.

Characteristically, the French *savant* was not content to use the Guidonian hand merely as a memory device, but transformed it into a location for his all-embracing theories of universal harmony. His mission was to rework the mathematical foundations of music laid down by Pythagoras in order to accommodate polyphony and the new modes of consonance and dissonance, correlating ancient and modern theories. To accomplish this, he brought his considerable sophistication in advanced mathematics to bear on the physical processes in vibrating strings and bodies that generate sounds.

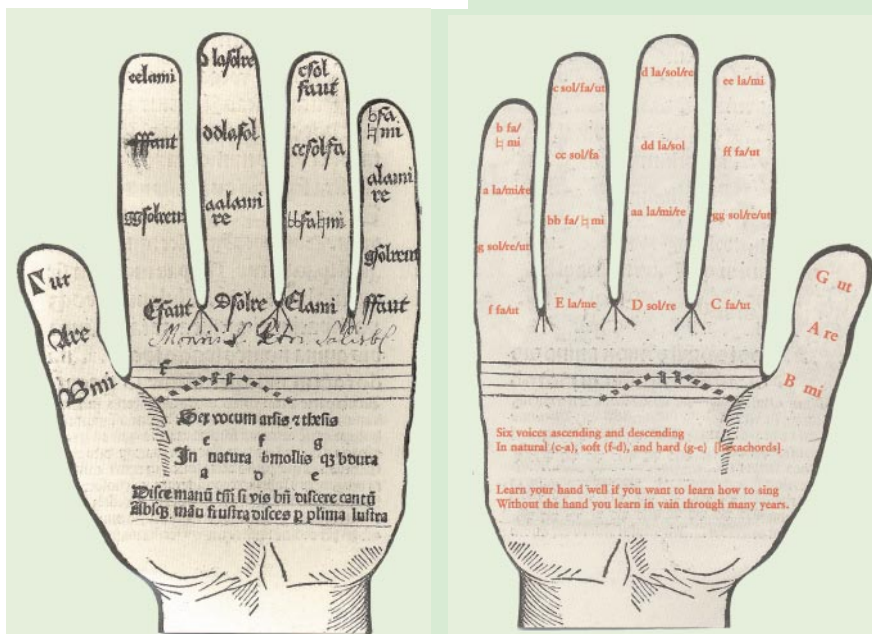
In his illustration of the harmonious hand, which appears twice in the second volume of his 1636 *Harmonie Universelle. Traité de la Voix et des Chants*, he shows on the left "the tetrachord divided into 12 sounds or 12 strings". Above, he demonstrates "the same divided into 9 strings". At the foot of the page he places "a composition by the same author [that is, Mersenne himself] in 8 parts". The text could not be more fitting: "Behold how good and how pleasant it is for brethren to dwell in unity."

Mersenne was a supreme representative of that philosophical, scientific and aesthetic tendency that aimed to establish a kind of 'unified field theory', in which every level of order in the universal and world systems was representative of the same underlying structures. To discern the harmonic architecture of these deep structures, the natural philosopher needed to divine the most abstract subtleties of mathematics and the actual properties of physical things. In this Mersenne was at one with the mathematician and astronomer Johannes Kepler.

The detailed components of the particular unity sought by Mersenne, not least the musical elements inherited from antiquity, did not survive later scrutiny. But his underlying ambition to place data from scientific experiment within the embrace of an overarching theory of mathematical organization is still recognizable as a potent motivation in modern physics.

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The plate of the "Guidonian Hand" is on view at the exhibition "Writing on Hands: Memory and Knowledge in Early Modern Europe" at the Folger Shakespeare Library, Washington DC, until 4 March 2001.



Gained in the translation

Scientific knowledge is enriched as it moves between languages.

Scott L. Montgomery

The word is no less central to science than it is to literature, philosophy or religion. Densely textual since its very beginnings, Western science could not exist without language, and resides largely within its domain. It is language, after all, that allows knowledge to find its cast and community, to be recorded, debated, shared and advanced.

Many implications flow from this simple truth. Among the most fertile is that, as with other areas of knowledge, science has been a mobile form of understanding. We say that science advances, but it also moves — across linguistic, cultural and temporal boundaries. And this movement has in turn often been integral to scientific advance, owing to the adaptations and additions made by different peoples at different times. Transfers of science have been critical to the building of societies, those we call modern most of all, because the acquisition of technical knowledge such as Arabic numerals, Newtonian physics or the periodic table, has led to many new powers over material existence. By any measure, therefore, movements of science are a significant historical process.

Translation renders knowledge mobile. The task of the scientific translator, no less than his or her literary relative, has been to create new texts, to multiply sources into new languages, and thereby to produce new 'originals'. Over time, translation itself has built a great scientific library, ever more enriched, accessible. Although we may think of scientific translation as literal, mechanical work, this has never been the case. The reasons for this are complex, but have much to do with the lack of exact one-to-one correspondence among languages. Translating science always involves interpretation, the remaking of an original. If it did not, machine translation would have long ago rendered the scientific translator vestigial or extinct.

Before the last century, no standards for

accuracy or methodology existed. Translators went about their work in whatever ways they deemed useful. Decanting scientific works involved both oral and written exchange, at times simultaneously; it was performed by individuals, by teams, even by groups. Its sources included original texts, corrupted and abridged texts, partial or reconstructed texts, even fabricated texts; resulting translations therefore represented material partly or wholly rewritten, beautifully adapted, ideologically reconstituted, or so literally repoured as to be nearly incomprehensible. Works of great importance, such as Ptolemy's *Almagest*, were usually rendered several times into each new language, by different hands, often with numerous additions, deletions, reorganization, interpretive commentary and more, all in the name of furthering their utility.

Ptolemy's great work, in fact, is an excellent example. Its very title, which means 'the greatest', is an Arabic-Latin replacement for the original Greek *Mathematica Syntaxis* (*Mathematical Treatise*), revealing centuries of added reverence. Mathematical astronomy in the West is inconceivable without what is often called "the Greek heritage in science", with the *Almagest* at its helm.

In fact, the *Almagest* did not enter Latin until quite late, as part of a vast translation effort that helped comprise the so-called Twelfth Century Renaissance. Roman society found most Greek science too difficult and abstruse. In early Byzantium, purges of 'pagan learning' forced this knowledge eastward, first in the care of Nestorian Christians, and then into the great commerce of learning of the Near East, where, between the fifth and seventh centuries AD, it gained new versions in Syriac, Pahlavi (ancient Persian) and Sanskrit. Parts of the *Almagest* were adapted by Indian astronomers into the siddhanta tradition, with star positions updated and poetic forms added. All this then came within the great intellectual embrace of Islam, whose Abbassid rulers had the intellectual wealth of the region translated into Arabic. In the capable hands of Hunayn ibn Ishak, Thabit ibn Qurra and Abd al-Rahman al-Sufi, *Almagest* astronomy gained still new corrections, refinements, commentary and a magnificent artistic dimension in drawings of the constellations.

It was this total body of work, then, that Europe chose to adopt and adapt. Between roughly 1120 and 1240 AD, the great library of



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The 'greatest': Ptolemy's work has been translated into a host of languages.

science in Arabic was transferred into Latin, producing a 'renaissance' at least as epochal as that several centuries later in art and literature. It was no accident, therefore, that the university arose during this same period, for it required just this kind of institutional invention to metabolize the flow of nutrient texts into Europe — the Greco-Syriac-Pahlavi-Hindu-Arabic tradition that included Euclidean geometry; Arabic algebra, optics and medicine; *Almagest* astronomy; Aristotelian thought and much more.

It was this period, after all — this naissance (birth) — of textual possession, that made possible so much that came afterward, including the Scientific Revolution. This was when Western knowledge grew beyond all measure, not merely in the rising shadow of gothic cathedrals, but in every library and classroom and makeshift laboratory in Europe. What entered Latin culture was much larger than 'Greek science'. Like its forebears, Europe adopted an accumulation of contributions, and became all the richer for it. At its historical base, Western science must be seen to stand upon many shoulders.

In the end, the age-old notion of *traduttore, traditore* (to translate, to deceive) seems thin, even irrelevant where the history of science is concerned. In truth, an enormous amount has been gained in the movement of technical knowledge among peoples. Scientists, too, are scholars of the inherited word. We are all enriched by the mobilities that translation has made possible. ■

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Western science must be seen to stand upon many shoulders.

The big picture

Sean B. Carroll

Macroevolution: "a vague term for the evolution of great phenotypic changes, usually great enough to allocate the changed lineage and its descendants to a distinct genus or higher taxon" — from D. J. Futuyma, *Evolutionary Biology* (Sinauer, Sunderland, 3rd edn, 1998).

Evolutionary change occurs on different scales: 'microevolution' is generally equated with events at or below the species level whereas 'macroevolution' is change above the species level, including the formation of species. A long-standing issue in evolutionary biology is whether the processes observable in extant populations and species (microevolution) are sufficient to account for the larger-scale changes evident over longer periods of life's history (macroevolution).

Outsiders to this rich literature may be surprised that there is no consensus on this issue, and that strong viewpoints are held at both ends of the spectrum, with many undecided. Traditionally, evolutionary geneticists have asserted that macroevolution is the product of microevolution writ large, whereas some palaeontologists have advocated the view that processes operating above the level of microevolution also shape evolutionary trends. Is one of these views wrong, or could they both be right?

One obstacle to a more unified, multi-disciplinary view of evolution is the vague meaning of the term macroevolution, and its different connotations in different disciplines. This is not merely a matter of semantics: scientific and broader public issues are at stake. The origins of major innovations and the underlying causes for the radiation of forms during various episodes in life's history are among the most interesting and challenging questions in biology. We must know whether, as G. G. Simpson asked, macro-

evolution differs fundamentally in kind or only in degree from microevolution. Furthermore, one of the latest political strategies of the creationist faction in the United States is to 'accept' microevolution but to bar macroevolution from the classroom on the misguided grounds that aspects of macroevolution are controversial and that therefore its scientific foundation is 'unproven'.

My argument has three parts. First, that the concept of macroevolution is better clarified when broken down into its two major component parts: phyletic and morphological evolution. Second, these two components are governed by at least partly distinguishable mechanisms. And third, because there is no evidence that the intrinsic genetic and developmental mechanisms underlying morphological evolution differ across any evolutionary scales, the distinction between these scales is really only descriptive, not mechanistic.

Deconstructing macroevolution

'Macroevolution' is a different concept for different kinds of biologists. For a palaeontologist, the fossil record frames a picture of the origins and extinctions of species, that is, phyletic evolution. For a developmental biologist or population geneticist, macroevolution is generally synonymous with morphological evolution, essentially independent of timescale or phylogenetic context. These different conceptual frameworks inspire different research agendas in the search for mechanistic explanations at different levels of biology.

One of the evolutionary phenomena for which the mechanistic discontinuity between macroevolution and microevolution has most often been asserted is the burst of innovation and diversification associated with major radiations of forms — for example, the dramatic phyletic and morphological evolution seen in the explosive Cambrian radiation of animal phyla. Explanations for the Cambrian patterns of phyletic evolution tend to focus on extrinsic environmental and ecological factors as catalysts for the rapid diversification of clades (sets of species each descended from a common ancestral species). There is also currently much interest in identifying the intrinsic genetic and developmental mechanisms for the morphological diversification of taxa in the Cambrian.

The crucial question is whether there is any evidence that distinct macroevolutionary mechanisms affect morphological or phyletic evolution. There are no reports of higher-level genetic mechanisms (genome rearrangements or 'macromutations') distinct from microevolutionary genetic mechanisms underlying speciation, the large-scale mor-

Macroevolution

"Many geneticists assert that macroevolution is the product of microevolution writ large, but some palaeontologists believe that processes operating at higher levels also shape evolutionary trends."

phological diversification of various body plans, or the origin of major innovations. On the contrary, an unexpected degree of genetic similarity exists between morphologically and phylogenetically divergent taxa, suggesting that the distinction between macro- and microevolution in terms of morphological change is descriptive, not mechanistic.

On the other hand, the differential success of some clades has led to the proposal that there are properties of species that make them, as units, subject to selection (for example, occupancy of a certain ecological zone). The controversial idea of species selection differs from the microevolutionary view of natural selection in regarding variations between species, rather than characteristics of individuals within a species, as the basis for evolutionary change. One major difficulty with species selection is the requirement for selection to act, with equal intensity and no differential effect, on all individuals across a species' geographical range. Should these (some would say unlikely) conditions not be met, species selection breaks down into essentially conventional microevolutionary processes.

Macro- = Micro- = Evolution

The 'big picture' of evolution continues to grow, with diverse disciplines addressing biological mechanisms across many levels of organization (molecules, organisms, populations) and timescales. The subdivision of evolution into two scales no longer reflects our understanding of the unity and diversity of evolutionary mechanisms. However, more important than redefining macroevolution is recognizing that discipline- or scale-bound considerations of only one component of evolution, or of solely extrinsic or intrinsic mechanisms, are inadequate. Long-standing boundaries between evolutionary disciplines are dissolving, to allow richer concepts of evolution to emerge. ■

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Micro or macro? A trilobite fossil.

The dark side of aerosols

Meinrat O. Andreae

According to new modelling calculations, black carbon in the atmosphere exerts a large warming influence on global climate. Curbing emissions of this pollutant may be advisable both on climate and on human health grounds.

For a while, it looked so simple: greenhouse gases warm the Earth and sulphate aerosols cool it down. Because aerosol particles in the atmosphere scatter sunlight back into space, they reduce the amount of energy that the planet absorbs, keeping it cooler in much the same way as a coat of light paint keeps a car cooler than does dark paint. But this simplistic view ignores the fact that aerosol particles in the real world are not white but grey, mostly because they contain soot particles from the incomplete combustion of fuels. And the darker grey the particles are, the more solar energy they can absorb, heating the atmosphere. On page 695 of this issue¹, Mark Z. Jacobson draws our attention to that dark side of atmospheric aerosols — he proposes that the warming effect from black carbon in aerosols may balance the cooling effect of the sulphate component, the largest single contributor to aerosol cooling.

It has been known for quite some time that aerosols can absorb solar radiation, and that their radiative effect — cooling or warming — depends on the proportion of light scattered to that absorbed². But although light scattering by aerosols can be measured and modelled fairly accurately, the assessment of the radiative effects of black carbon has remained a challenge for both modellers and experimentalists. It is not uncommon for black-carbon measurements with different techniques to vary by a factor of two, mostly because black carbon is actually a mixture of graphite-like particles and light-absorbing organic matter. Many techniques are not selective enough to distinguish between these components.

Direct optical measurements of aerosol light-absorption are also problematic³. They typically start with the collection of aerosols on a filter; as a result, the optical properties of the particles are changed by contact with the filter material, or with one another. Only one instrument, the photoacoustic spectrometer, directly measures the light absorption of particles suspended in the atmosphere, but it has not yet been widely used. Finally, lack of a generally accepted calibration procedure is a problem affecting all measurements of black carbon and light absorption.

These methodological difficulties have made it difficult to answer the key questions about black carbon: where does it come from, how much of it is emitted, how is it



Figure 1 Vehicle fumes, a source of black carbon. Knowledge of the global amount and distribution of black carbon is poor, and severely hampers attempts to assess its radiative effect.

distributed in the atmosphere and what are its radiative effects? We know that the main sources are the burning of biomass and fossil fuels (Fig. 1), but there are large uncertainties in estimating the magnitude of either of them. Without quantitative tools to determine aerosol absorption worldwide by remote sensing, our knowledge of the global distribution of black carbon remains inadequate. An illustration is the 1999 discovery of the 'Indian plume' by the INDOEX project⁴, which showed massive amounts of black carbon being emitted from southern Asia, adding maybe as much as 25% to the previously known global sources of this pollutant.

Clearly, then, our poor knowledge of the amount and whereabouts of atmospheric black carbon severely hampers our ability to assess its radiative effect. Given this situation, Jacobson¹ works with the best current knowledge or assumptions, imperfect though they may be, about the sources of black carbon. He uses them as input for a global model that predicts black carbon's global distribution. But to derive the resulting effects on climate, he finds that precise knowledge is needed about exactly how black carbon is mixed with other particles in the aerosol.

The mixing state that Jacobson considers most plausible is that of a black-carbon core

aggregated with other aerosol components. For this, his model calculates a global-mean radiative warming of 0.55 W m^{-2} , as compared with the International Panel on Climate Change estimates⁵ of 0.47 W m^{-2} for CH_4 (methane) and 1.56 W m^{-2} for CO_2 (carbon dioxide). If research investment were to be scaled by climate impact, these figures would suggest that resources at the level of about one-third of those now devoted to carbon-cycle research should go into black-carbon studies — a staggering thought!

But is it reasonable to compare the present-day effects of black carbon, which has a lifetime of about a week, to that of the long-lived greenhouse gases, which have lifetimes of decades or centuries? The accepted way to compare the climate impact of a chemical species emitted to the atmosphere is using 'global warming potentials', which relate the species' radiative effects over a given time to those of CO_2 . This may be a difficult exercise to carry out for black carbon, given that its lifetime, and also its distribution, are so different from those of CO_2 . But it is clear that black carbon's impact is most important on short timescales, whereas those of CH_4 and CO_2 reach much farther into the future.

In this short lifetime, however, lies a possible advantage: if we could somehow

stop emitting black carbon today, it would be gone from the atmosphere in a week or two. This is only a thought experiment, of course: in the real world, the black-carbon burden of the atmosphere can only decrease as fast as human society manages to curb black-carbon emissions.

Beyond these climate-related issues, black carbon also differs from the greenhouse gases by being an air pollutant that harms human health. This combination of characteristics — significant effects on climate, short lifetime and toxic consequences — has led Hansen *et al.*⁶ to propose that consideration should be given to taking urgent action to reduce black-carbon emissions.

Diabetes

Dialogue between muscle and fat

Morris J. Birnbaum

Knocking out a glucose transporter in fat cells also affects the uptake of glucose into muscle cells, which may prompt a rethink of the transporter's role in diabetes.

One of the more troublesome stresses that organisms endure is the metabolic demand imposed by unpredictable cycles of feeding and starvation. Mammals solve this problem by using complex macromolecules to store energy efficiently. So, after a meal, simple nutrients such as sugars are taken up into liver, muscle and fat cells. There they are converted into complex carbohydrates, protein and fat, which — with varying degrees of success — store potential energy in a minimal amount of space. One of the forces that drives this storage process is insulin, a hormone released by pancreatic β -cells in response to glucose and amino acids.

Insulin promotes the uptake of glucose into muscle and fat cells, and suppresses the production of glucose by the liver. But it is now becoming clear that not only do β -cells communicate with the organs that store energy. These organs also talk among themselves. On page 729 of this issue¹, Abel and colleagues lend weight to this idea, with their surprising discovery that a genetically engineered defect in fat cells also affects how liver and muscle cells respond to insulin.

One of the reasons why scientists are so interested in nutrient storage and mobilization is that this process is disrupted in diabetes mellitus. Ninety per cent of diabetics have type 2 diabetes, a significant feature of which is 'insulin resistance' — defects in the responses of muscle and liver cells to insulin². Obesity is often, if not always, a prerequisite for type 2 diabetes. So the concept has developed that, somehow, an increase in fat (adipose) tissue in genetically susceptible people results in insulin resistance in muscle and liver cells.

But an essential first step is that we make every effort to reduce uncertainties about this pollutant's sources, its chemical and physical properties, and its radiative effects in the atmosphere.

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marked effects on insulin's action in muscle cells⁷. It seems that adipose tissue is more than capable of telling other energy-storing organs what to do. Moreover, there are also ample data to suggest that the liver can sense the level of sugar absorbed from a meal, and convey this information directly to muscle cells without depending on insulin^{8,9}.

Abel *et al.*¹ have now used genetic techniques to disable insulin-stimulated glucose uptake in fat cells in mice. Intriguingly, although the genetic defect is specific to fat cells, muscle and liver cells no longer show the usual responses to insulin, either (Fig. 1). The explanation is probably that the altered fat cells secrete a factor that travels in the blood to the muscles and liver. This theory is supported by the fact that muscle removed from the engineered mice — isolating it from factors secreted by the fat cells — responds appropriately to insulin¹. The authors excluded many of the usual suspects — leptin and tumour-necrosis factor- α , for example — as the culpable fat-secreted factors in this case. So one of the most important implications is that fat cells must use yet more messengers to communicate with liver and muscle cells.

How might the secretion of this unknown factor be controlled? Abel *et al.*'s work offers some hints. In the 1980s, the idea emerged that insulin increases the uptake of glucose into a fat or muscle cell by causing a dormant, intracellular pool of a glucose-transport protein to be redistributed at the cell's surface¹⁰. The protein is the specialized sugar carrier GLUT4, which is greatly enriched in muscle and fat cells. Abel *et al.* impaired insulin-dependent glucose uptake in their mice by disrupting the *GLUT4* gene in fat cells, and this disruption must somehow be responsible for the general insulin resistance seen in the mutant rodents.

But this study has another implication that might be important in understanding

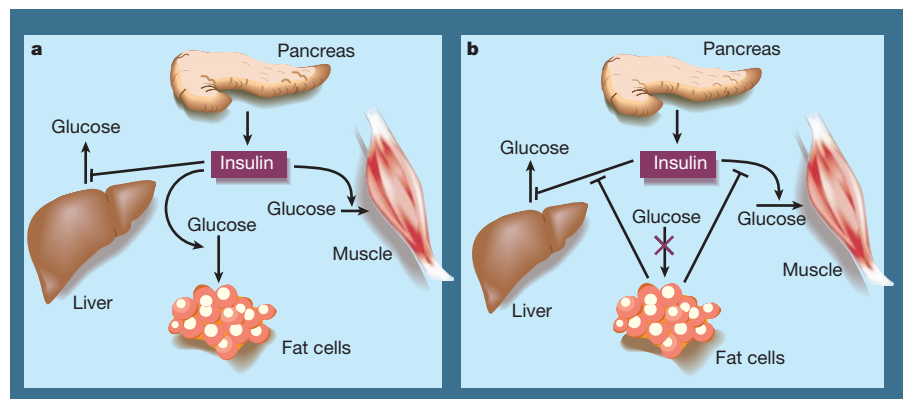


Figure 1 Communication between fat, muscle and liver. a, Normally, insulin is secreted by pancreatic β -cells after a meal. Insulin accelerates the uptake of glucose into muscle and fat cells, and suppresses the production of glucose by the liver. b, Abel *et al.*¹ have used genetic techniques to disable the ability of fat cells to increase their uptake of glucose when they are exposed to insulin. Surprisingly, this fat-cell-specific defect also results in a more generalized resistance to insulin — muscle and liver cells no longer respond appropriately. This is presumed to be because the fat cells produce a factor that circulates in the blood and blocks the response to insulin in the muscles and liver.

type 2 diabetes. After the discovery of GLUT4, there was much enthusiasm for the idea that decreases in the cellular content of this protein might be responsible for the insulin resistance seen in diabetes. Indeed, the abundance of GLUT4 in different muscle types roughly correlates with the ability of those muscles to take up glucose¹⁰. But it became clear that, although GLUT4 levels are lower in fat cells from diabetic people, the amount of GLUT4 in muscle cells in such people changes little². As muscle accounts for most of the mass of insulin-responsive tissue, any physiologically significant impairment in glucose disposal *in vivo* must be contributed mainly by muscle. So the GLUT4 idea was abandoned. But Abel *et al.*'s new data¹ again raise the provocative theory that changes in GLUT4 expression contribute to diabetes.

The next steps are clear. First, it is important to pin down the critical but elusive fat-cell products that impair insulin's ability to regulate metabolism in muscle and liver cells. Second, it will be equally interesting to

find out how inhibiting insulin-dependent glucose uptake in fat cells transmits the signal that ultimately alters the secretion of those products. An attractive idea is that this process represents another example of intracellular 'glucose sensing'. This phenomenon was once thought the exclusive province of the pancreatic β -cell. But it is clearly much more widely used. ■

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Astronomy

The age of the Universe

Christopher Sneden

Dating the Universe has always been a tricky business with unsatisfying answers. Astronomers now have a better clock, based on radioactive uranium, that puts the age at around 12.5 billion years.

How old is the Universe? This simple and fundamental question has dominated much of astronomy for centuries. Many different techniques have been applied to the problem, including observations of the expansion rate of the Universe, implied by the velocities of distant galaxies, and of the luminosity of the faintest white dwarf stars, which are dying embers of their former selves. But these methods cannot give direct measurements of age — they all rely on assumptions about the nature of the objects being observed. A better approach, radioactive cosmochronometry, relies on measuring the abundance of radioactive thorium found in stars. On page 691 of this issue, Cayrel *et al.*¹ report the discovery of radioactive uranium in a very old star called CS31082-001, resulting in a major advance for this method. Radioactive cosmochronometry may some day prove to be the anchor for all other estimates for the age of the Universe.

Almost all chemical elements are formed or synthesized by nuclear fusion reactions in the hot and dense interiors of stars or in supernovae. Fusion cycles, such as the conversion of hydrogen to helium and then to carbon, give off energy, which provides the outward pressure needed to stabilize stellar interiors against the inward pull of gravity

and the light output of the stars. Progressively heavier nuclei require more energy to form and fusion becomes increasingly difficult. Nuclei of iron are the most tightly bound of all, with nuclei heavier than iron becoming unstable and difficult to create by fusion. So massive stars are fuelled by fusion until they develop an iron core, at which point unbalanced gravitational forces cause the core to implode, sucking in the outer layers until they meet in the middle and rebound, releasing energy — this is a supernova.

The extreme temperature and density of a supernova create huge but short-lived fluxes of neutrons. These fluxes yield extraordinarily neutron-rich nuclei that quickly rearrange themselves for greater stability. This mechanism, called neutron capture, is responsible for the production of the heavy, long-lived radioactive elements, most notably thorium (the half-life of ²³²Th is 14.1 billion years (Gyr)) and uranium (the half-life of ²³⁸U is 4.5 Gyr; ²³⁵U has a much shorter half-life and so is of less interest here). These elements are especially useful for astronomy because their half-lives are considerable fractions of current estimates of the age of the Universe as determined from other techniques. These estimates currently span the range 9–16 Gyr (for example, see ref. 2).

A high-mass star born early in our Galaxy's history would end its typically short life in a supernova explosion. If that supernova spewed out elements produced by neutron capture into the interstellar medium, some would be radioactive thorium and uranium, and many of those atoms should still be around today, because of their long half-lives. The next generation of stars can sweep up supernova ejecta and we should be able to detect them in their atmospheres by measuring the absorption spectra of the ionized species.

Happily, these elements have been found in stars that reside in the outer regions of the Galaxy — the Galactic halo. Here there are demonstrably old stars that have much less iron (and similar metals) than our relatively young Sun. In some cases, these very metal-poor stars have less than a thousandth of the Solar metal abundance — they have absorbed little supernova ejecta because, when they were born, very few element-donating stars had died. It is difficult to find metal-poor stars, and taking a census of our Galactic halo at moderate photometric and spectroscopic resolutions³ was a necessary first step.

Ionized species of the rare-earth metals from supernova ejecta are easily detected in metal-poor stars that are enriched in the neutron-capture elements. However, thorium is less prominent, and its abundance in most stars has to be derived from a single transition that is contaminated with other absorbing species⁴. Despite this, thorium has been detected in several metal-poor stars, but in smaller amounts than in the stars in the vicinity of our Solar System, implying that the metal-poor stars are substantially older. Detailed thorium and neutron-capture abundance studies^{5,6} suggest that the synthesis of thorium occurred some 14–16 Gyr ago, but the error estimates on this value are frustratingly large, about 4 Gyr.

Cayrel *et al.*'s studies of the star CS31082-001 could bring us closer to finding out the age of the Universe. This star, serendipitously discovered in a large high-resolution spectroscopy study of very metal-poor stars, has about a thousand times less iron than the Sun. But its neutron-capture elements still exist in high quantities, so their absorptions stand out prominently in this star's spectrum. The authors detected not one but eleven transitions of thorium in CS32081-001. And most importantly, an ionized uranium transition is detected for the first time in a metal-poor star.

The importance of having abundances of both thorium and uranium in a single star cannot be overstated. It is difficult to predict the production of either of these elements by themselves in supernovae and a lively debate^{7,8} has centred on how to turn an observed thorium abundance into a reliable age estimate. But it is relatively easy to predict

the relative production of thorium to uranium because these elements are separated by only two atomic numbers. And the different decay rates of ^{232}Th and ^{238}U ensure that the abundance ratio of these two elements will be a sensitive function of their age. Cayrel *et al.*¹ propose that the neutron-capture material in the atmosphere of CS31082-001 has an age of 12.5 Gyr with an uncertainty of 3.3 Gyr, a more accurate estimate of the age of the Universe. Further analysis of the whole range of neutron-capture elements in this star will refine this age estimate, narrowing the uncertainty.

We now know of a handful of stars born early in our Galaxy's history that are anomalously enriched in radioactive thorium, and at least one with uranium. We may expect

to find more examples of such stars, as our surveys of the Galactic halo with the new generation of very large telescopes is just beginning. With new discoveries, more age estimates will be found, further nailing down the exact age of the Universe.

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Evolution

Infectious speciation

Michael J. Wade

The bacterium *Wolbachia* has strange and wonderful effects on reproduction in its many invertebrate host species. In effect, the creation of new species can now be added to the list.

For a new species to arise, a single population must somehow be split into two reproductively isolated populations that cannot interbreed. Such reproductive isolation usually stems from genetic incompatibility. It is easy to see how that arises when a geographical barrier divides one population of an organism into two, which then diverge genetically. On page 707 of this issue, however, Bordenstein, O'Hara and Werren¹ show that in two species of parasitoid wasp it is microbial infection that is the barrier to gene exchange.

The microbe concerned, *Wolbachia pipiens*, is a member of a highly diverse group of bacteria that is thought to include the ancestor of the mitochondrion — the powerhouse of multicellular organisms that was originally free-living. *Wolbachia* are endosymbionts, living inside the cells of certain host organisms, and like mitochondria they are almost always inherited through the maternal line. Their host range is broad, for the bacteria are found in association with about 20–75% of the insects, crustaceans, mites and nematode worms that have been surveyed with molecular markers^{2,3}. Such is the range of effects of the microbe on its host — positive and negative — that it is not always possible to characterize *Wolbachia* simply as a mutualist, symbiont or pathogen.

Among the variety of reproductive anomalies caused by *Wolbachia* is the phenomenon of cytoplasmic incompatibility (Fig. 1), which results in the failure of infected host males and uninfected host females to produce offspring. *Wolbachia*

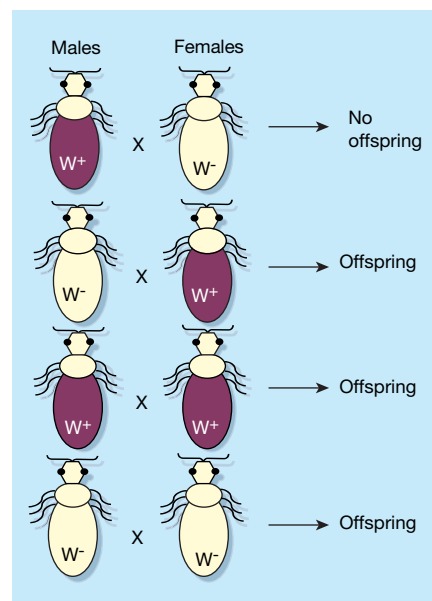


Figure 1 *Wolbachia* and cytoplasmic incompatibility. Cytoplasmic incompatibility means that when a male host infected with *Wolbachia* (W^+) mates with an uninfected female (W^-), no offspring are produced. All other matings are fully compatible and result in the production of offspring. The consequence of this system is that the maternally transmitted *Wolbachia* tend to spread through the host species.

residing in host males are not typically transmitted to offspring, but they eliminate competing uninfected maternal lineages from the host population by their incompat-



100 YEARS AGO

Dr. R. A. Daly, of the Department of Geology and Geography of Harvard University, is endeavouring to organise a geological and geographical excursion in the North Atlantic for the summer of 1901. Conditionally on the formation of a sufficiently large party, a steamer of about 1000 tons, specially adapted for ice navigation, and capable of accommodating sixty persons, will leave Boston on or about June 26... The main object of the voyage will be to offer to the members of the excursion party opportunity of studying the volcanic cones and lava-fields, the geysers, ice-caves and glaciers of Iceland, the fiords and glaciers of the west coast of Greenland, and the mountains and fiords of Northern Labrador... A hunting party may take part in the expedition; it could be landed for a fortnight or three weeks in Greenland and for about the same period in Labrador. From *Nature* 7 February 1901.

50 YEARS AGO

Surprisingly little of the information obtained with microscopes has been quantitative; most observers are content to sit at the microscope and regard the image, or to photograph it. Theoretically, it is possible to scan the image or its photograph mechanically; but this has seldom been done in practice. The whole method of obtaining resolution by lenses involves so much loss of light, lack of control of contrast, and other difficulties, that it is difficult to provide a good display or method of scanning. Some of these difficulties can be avoided by using a wholly different means of obtaining resolution and amplification. The essence of the problem of resolution is to separate in some way the light passing through very close regions of an object. The conventional microscope does this by using refraction by lenses to separate the light from neighbouring regions. An alternative method is to use the lens system the other way round, namely, to produce a minute spot of light. Discrimination between neighbouring points is then produced by passing the light through them at different times by making the spot scan it. After passing through the preparation, the spot is made to fall on a photocell, with subsequent amplification and display as required. Such a flying-spot microscope depends on scanning different parts at different times, and will only give accurate information about objects that are stationary or moving only at a rate of a different order from that of the spot. From *Nature* 10 February 1951.

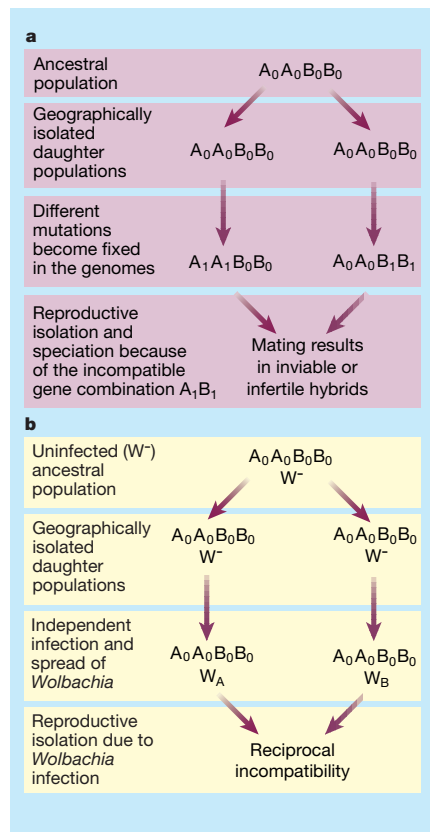
ible matings. So the bacteria in males are essential to the spread of their maternally transmitted relatives through the host population.

Typical cytoplasmic incompatibility falls short of speciation because the barrier to reproduction between infected and uninfected populations works only in one direction, not reciprocally. Although females of the uninfected host population cannot interbreed with males of the infected host population, the reciprocal cross is fully fertile. But there is evidence^{4,5} that different genetic strains of *Wolbachia* can cause reciprocal, two-way reproductive isolation between host populations in some parasitoid wasps, mosquitoes and fruit flies⁶. This observation has led some evolutionary biologists to speculate that *Wolbachia* might be an agent of infectious speciation^{6,7}.

Such speculation is controversial, for two reasons. First, it is widely accepted that, when two host populations become reproductively isolated, so do the populations of their respective endosymbionts. Hence, in a process called co-speciation, a host may cause subsequent speciation of its endosymbionts, an explanation suggested for the genetic divergence of strains of *Wolbachia*⁸. The hypothesis of infectious speciation turns this view on its head. Second, so the theory goes, speciation occurs when reproductive isolation arises as the incidental by-product of the gradualistic, genetic divergence of two populations. Microbial speciation, in contrast, might be comparatively rapid (as seen for instance in polyploid or hybrid speciation in some plants⁹), and could occur without any genetic evolution of the host. Polyploid speciation occurs through a doubling, or more, of chromosome number.

Bordenstein and colleagues¹ provide evidence that microbes have acted faster than genes in producing reproductive isolation between the wasps *Nasonia giraulti* and *N. longicornis*; this can be taken as the first stage of speciation. First, the authors showed that each wasp harbours a genetically distinct strain of *Wolbachia* that causes cytoplasmic incompatibility with the other uninfected host species. They then used antibiotics to create an uninfected strain of each host species and demonstrated that in *Wolbachia*-free wasps there are no genetic barriers in first- or second-generation hybrids to free interbreeding between the two wasps.

How might these findings fit into a standard genetic model of speciation, as shown in Fig. 2a? In this model, incompatible gene combinations (such as A_1B_1) cause sterility or inviability of offspring, and so speciation. Events begin with an ancestral species, $A_0A_0B_0B_0$, that becomes split by geological events into two geographically isolated daughter populations. The evolutionary forces of mutation, random genetic drift



and natural selection operate independently on each daughter population. Eventually, one gene undergoes mutation to allele A_1 , and becomes fixed in one daughter population, while a second mutation, to allele B_1 at the other gene, becomes fixed in the second daughter population. The two daughter populations become reproductively isolated because matings between them result in the A_1B_1 deleterious gene combination. In this classic model, genetic barriers to reproduction and genetic exchange, and so speciation, arise as a by-product of local, gradual evolution.

Microbially driven speciation could occur in much the same way, stemming from cytoplasmic incompatibility between two different strains of *Wolbachia* infecting the same host species (Fig. 2b). Here, however, infectious transmission of incompatible *Wolbachia* strains, one in each daughter population, replaces the incompatible gene combinations. Predatory mites and parasitoid wasps are the most likely candidates for spreading *Wolbachia* between different species of host. Previous cases of reciprocal cytoplasmic incompatibility have been between species pairs, which also exhibited evidence of genetic barriers to gene exchange. Whenever both are present, it is difficult to determine which — the incompatible gene combinations or the microbes — came first. The report by Bordenstein *et al.* provides evidence that, at least in this case, microbially induced reproductive isolation preceded genetic isolation.

Figure 2 Genetic and infectious models of speciation. a, A standard genetic model in which the initial state is an ancestral population of a species that is homozygous at both of two gene loci, and so is $A_0A_0B_0B_0$. Following geographical isolation, each of two daughter populations is gradually modified as new alleles (A_1 and B_1) arise by mutation and then become fixed in the genome by random genetic drift and natural selection. Because the A_1B_1 gene combination causes complete inviability or sterility in hybrids, the daughter populations are new, descendant species. b, Infectious speciation, which parallels the genetic model. The initial state is an ancestral species, W^- , not infected with *Wolbachia*. Two daughter populations arise which have become infected by different strains of *Wolbachia* (A and B) after transmission from a parasite or parasitoid. The different strains then become fixed in each genome by cytoplasmic incompatibility. Reciprocal cytoplasmic incompatibility between W_A males and W_B females, and W_B males and W_A females, prevents hybridization, so in effect the daughter populations are new species even though they remain genetically identical to one another and to the ancestor.

How common might infectious speciation be? It is not possible to draw a conclusion from this single example — which has of course to be contrasted with the many examples of genetic speciation¹⁰. But there are several reasons why it is unlikely to happen often. First, incomplete cytoplasmic incompatibility (where incompatible crosses produce some progeny instead of none) seems to be more common than complete cytoplasmic incompatibility. Reciprocal but incomplete incompatibility is not a barrier to gene flow. Second, genetic models of *Wolbachia*-host coevolution indicate that the favoured trajectory is from complete to incomplete cytoplasmic incompatibility. Finally, we know little of the initial stages of *Wolbachia* infection in natural populations. When artificially introduced into new hosts, *Wolbachia* can be difficult to transmit¹¹. So the experimental results are consistent with the scheme outlined in Fig. 2b, but may not reflect the actual historical sequence of events.

Nevertheless, with the paper by Bordenstein *et al.*, host speciation can now be added to the list of modifications to reproduction caused by *Wolbachia* infection. Given the ubiquity of *Wolbachia*, infectious barriers to gene exchange may be much more common in the early stages of speciation than we realize.

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Astronomy

The day the solar wind nearly died

Mike Lockwood

On 11 May 1999, the density of the solar wind dropped almost to zero. Space scientists are now giving their first reports of this rare opportunity to study the complex relationship between the Sun and Earth.

The study of space is generally passive, as the input factors to an environment cannot be adjusted in a controlled manner to study one isolated mechanism, as they can in a laboratory. Instead scientists have to monitor all the inputs and try to disentangle the various effects that are taking place simultaneously. For instance, the Sun emits a continuous stream of ionized gas (containing mostly protons and electrons) called the

solar wind, which varies in concentration, flux, speed, temperature and composition. All of these factors affect the magnetosphere — the cavity formed by the Earth's magnetic field in the solar wind — and separating their various effects is difficult. This is why rare events such as the one centred around 11 May 1999 are so valuable. In this period, the solar wind remained completely normal except that its density plummeted to 5% of

typical values. The first studies from this period are now published in a special issue of *Geophysical Research Letters*¹.

When the density dropped, many aspects of the magnetosphere's behaviour were as scientists had predicted, which was a satisfying triumph for current theories. But the event also had some puzzling characteristics. Some of these are apparent in the data presented in these initial papers, although not all are commented on. Others aspects are so intriguing that further study is required.

Earth's magnetic field is confined to the low-density, high-field magnetosphere by the dynamic pressure of the solar wind on the side of the Earth facing the Sun, and by thermal pressure on the long tail that trails away from the Sun (Fig. 1). Both these pressures depend on the concentration of the solar wind, so the magnetosphere grew to exceptionally large dimensions (100 times its typical volume) as the solar wind decayed. Another feature was the appearance of highly energetic flows of electrons parallel to the direction of the magnetic field in the vicinity of Earth. These so-called 'strahl' electrons (red arrows in Fig. 1) are continuously emitted by the Sun but their flow is usually disrupted by the solar wind, making their fluxes

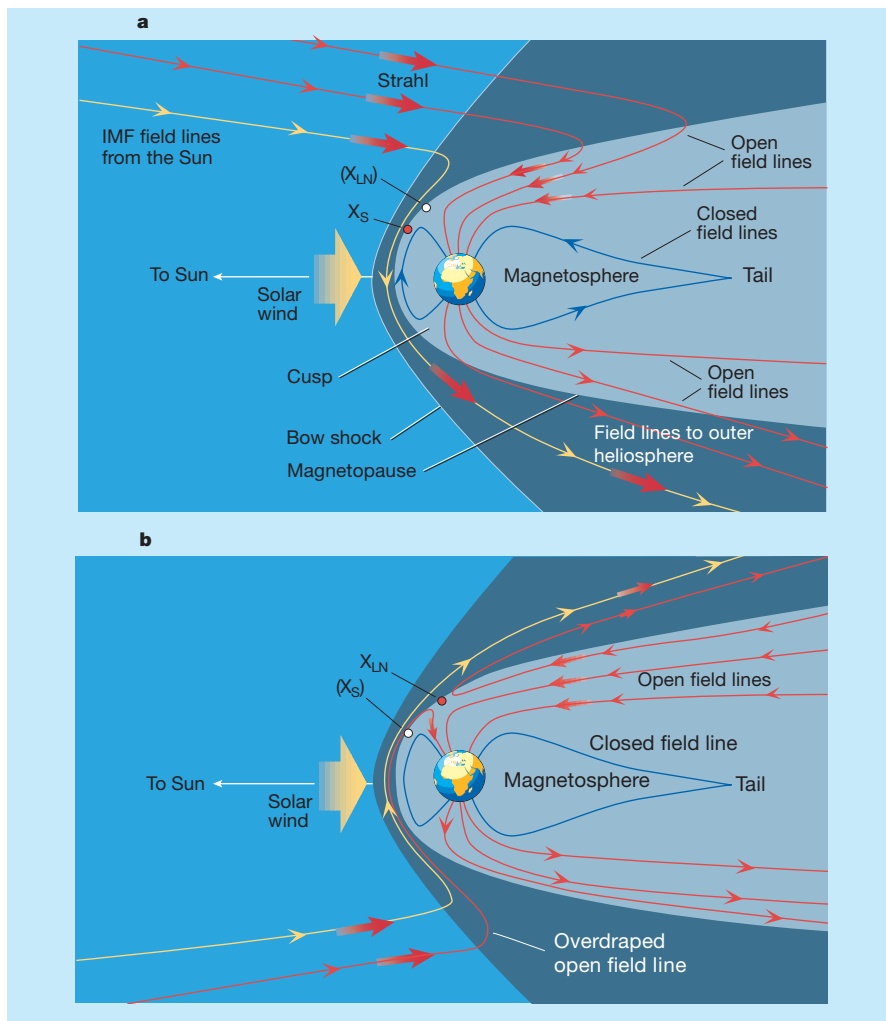


Figure 1 Earth's magnetosphere and the solar wind. a and b show two possible ways in which the interplanetary magnetic field (IMF) can interconnect with Earth's magnetospheric field. a, New open field lines (red lines) are produced at a reconnection site X_S and solar wind energy is directly deposited in the inner magnetosphere and upper atmosphere, as well as being stored in the tail of the magnetosphere because open field lines accumulate there. b, Field lines that are already open are reconfigured by reconnection at X_{LN} , in this example in the Northern Hemisphere. In this instance, solar-wind energy is not added to the tail because no new open flux is produced. Closed field lines are shown in blue; unconnected IMF lines are yellow; strahl electrons are represented by red arrows. The magnetopause is the boundary between the magnetosphere and the solar wind, and the bow shock is the edge where the supersonic solar wind abruptly drops in velocity. The solar wind behind the bow shock (dark blue) is denser than the incoming solar wind (medium blue), whereas the magnetosphere (grey) is the least dense of the three regions. A study of Earth's magnetosphere during a period of exceptionally low solar-wind flux promises to explain the complex interplay between these two situations¹.

weak near Earth. One surprise in this event was how brightly the Earth's northern polar cap emitted X-rays in response to the strong strahl precipitation².

Because the flow of strahl electrons was so strong, the event provided a uniquely clear demonstration of 'magnetic reconnection' between the Earth's field and the interplanetary magnetic field (IMF; yellow lines in Fig. 1) — the part of the Sun's magnetic field that is carried by the solar wind. Magnetic reconnection occurs when oppositely directed magnetic fields come together and form a new configuration that results in particle energization. Reconnection produces 'open' magnetic field lines that directly connect the magnetosphere with interplanetary space. (In contrast, a 'closed' magnetic field line never crosses the magnetosphere's boundary, the magnetopause; Fig. 1.) Because the IMF was pointing away from the Sun during this event, open field lines in the Northern Hemisphere were connected directly to the Sun, whereas those in the southern polar cap were connected to the outer heliosphere — a region of space around the Sun that stretches well beyond Pluto (Fig. 1a). As a result, strahl electrons from the Sun flooded directly into the northern polar cap, but not into the south. This was an outstanding re-verification of an early prediction of reconnection theory³.

Closer examination of where the strahl electrons were seen revealed some surprises. Because they enter the magnetosphere by flowing along field lines, the strahl in the northern polar cap shows which of Earth's field lines are open. Satellites flying over the polar caps saw pairs of field-aligned currents that, at first sight, appeared to be the normal response to magnetospheric reconnection with the IMF. However, we would expect these to be on closed field lines and, although this was true for the currents near dawn in both hemispheres, near dusk in the Northern Hemisphere they were on open field lines. This can be explained by an extreme rotation of the low-altitude signatures of the reconnection site X_S (see Fig. 1a) from noon to dusk⁴. This is consistent with the solar-wind proton precipitation seen at dusk. Small shifts of this 'cusp' in local time had been predicted, but the magnitude of the shift in this case was a surprise, and may have arisen because the magnetic field in the outer regions of the enlarged magnetosphere was weak.

The field-aligned currents at dusk were found to be strong in the Northern Hemisphere, but entirely absent from the south⁴. It seems that this asymmetry cannot be due to the lack of sunlight in the winter (southern) polar cap because the dawn currents were not similarly suppressed. The IMF orientation observed, with components away from the Sun and northward, favours the

Northern Hemisphere for a second type of reconnection at a higher-latitude site such as X_{LN} in Fig. 1b (ref. 5). There is no signature of this in the southern polar cap, which offers an alternative explanation of the hemispheric asymmetry. The surprise is that both types of reconnection appear to have taken place simultaneously for an extended period^{6,7}. This may have been possible because the reconnection at X_S that generates the new open flux was shifted to the dusk flank, whereas the reconnection of already open flux at X_{LN} may have taken place nearer noon.

Comparison of the two models proposed in Fig. 1 may be reminiscent of a spot-the-difference competition; however, understanding how the relative importance of these two types of reconnection varies has practical implications. For example, spacecraft can be destroyed by energetic electrons produced in the inner magnetosphere using the energy extracted from the solar wind and stored in the magnetic field of the magnetospheric tail. This stored energy drops when the tail expands because of reduced thermal pressure of the solar wind. Only reconnection that generates new open flux (as in Fig. 1a but not in Fig. 1b) increases

the stored energy, and during this event the harmful electrons decayed away to very low fluxes and took longer than expected to recover to normal values.

The rarity of such solar-wind events, and the solar conditions that give rise to them, are discussed in the other papers in the special issue. The papers published so far have some promising proposals, but they add up to neither a coherent nor a comprehensive picture of the day the solar wind almost died. But they do suggest that this event may well help to answer unresolved questions about solar-terrestrial relations. ■

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Developmental biology

A macho way to make muscles

Olivier Pourquié

The 'mosaic' theory of development applies, to different degrees, to most animals. It owes its existence in part to a group of obscure marine invertebrates, which now take centre stage in the molecular age.

The development of many animal species — from insects to vertebrates — relies in part on material deposited in specific places in the egg by the mother. The first evidence for such localized 'maternal determinants' was provided in 1905 from classical studies¹ of muscle development in ascidians, a group of marine invertebrates. But the molecular identity of the muscle determinant remained elusive. On page 724 of this issue², Nishida and Sawada describe a likely candidate — a messenger RNA molecule that they call macho-1. Ascidians are back in the limelight, with a good chance of staying there.

During the seventeenth century, the popular 'preformationist' theory suggested that development consists simply of the growth of tiny but fully formed embryos contained in the sperm or egg. Preformationism did not survive the invention of microscopy in the eighteenth century and subsequent observations of developing embryos. But a more sophisticated version of the theory — the concept of mosaic development — was proposed in the nineteenth century³ and is still around today. According to this theory,

the egg contains, not a fully formed embryo, but rather a mosaic pattern of determinants that control the development of the particular embryonic cells that inherit them.

This concept, too, took a battering, in the late nineteenth century from experiments in which the cells (called 'blastomeres' at this early stage of development) of a four-cell sea-urchin embryo were separated from one another⁴. In contrast to the predictions of the mosaic theory, these separated cells developed into four larvae, instead of four partial embryos. So, like the fertilized egg, early embryonic cells exhibit 'totipotency' — the ability to give rise to all the different cells that make up an organism. Around the same time, however, similar studies showed that ascidian embryos behave as predicted by the mosaic theory: separation of ascidian blastomeres led to the development of just the embryonic parts normally fated to be formed by those cells⁵. Soon afterwards, evidence for localized maternal determinants in ascidians was provided¹.

Ascidians are marine invertebrate chordates. They develop as a tadpole with a body plan similar to that of vertebrates — a noto-

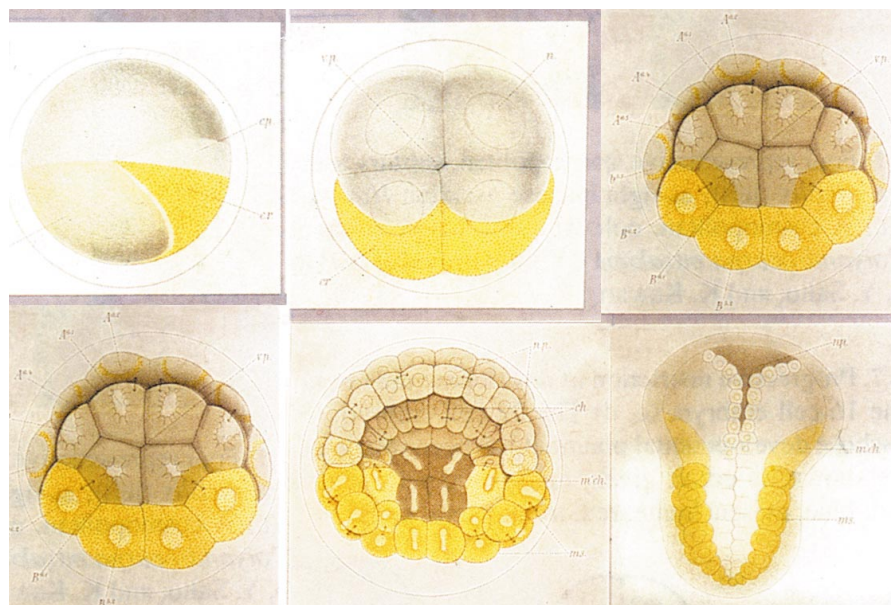


Figure 1 Stages in the early development of ascidians, as drawn by Conklin¹. The drawings show clearly the 'yellow cytoplasm' — a substance deposited in a specific region of the egg from the mother. The cells that inherit the yellow cytoplasm develop into muscle. Nishida and Sawada² suggest that the *macho-1* messenger RNA is the muscle determinant found in yellow cytoplasm.

chord, overlaid by a neural tube, runs along the back⁶. The tadpole comprises only 2,500 cells, 42 of which are muscle cells. As found for the nematode worm *Caenorhabditis elegans*, the pattern of cell division never varies in ascidian embryos, and researchers have determined the cellular genealogy up to the 110-cell stage. All of these features led to ascidians becoming a model organism for studying mosaic development. Complete mosaic development probably does not exist, even in ascidians or *C. elegans*. But mosaic patterns of maternal determinants are important, to different degrees, in the development of many animals, including frogs, fish and flies.

The first localized maternal determinant in an animal egg to be identified, back in 1905, was the so-called 'yellow cytoplasm'¹. The fertilized egg of the ascidian *Styela partita* is composed of differently pigmented subdomains. Conklin¹ followed the distribution of these domains during development, and observed that the cells that inherited the yellow cytoplasm invariably differentiated into muscles (Fig. 1). The role of this yellow cytoplasm, since termed myoplasm, was established by strategies such as cytoplasmic transfer experiments: cells that were not fated to form muscle did so when they were fused with fractions of blastomeres containing the myoplasm but lacking nuclei⁷.

But the question of what the yellow cytoplasm actually consists of went unanswered for nearly a century. Now, however, Nishida and Sawada² have identified, in the ascidian *Halocynthia roretzi*, a gene with all the characteristics of a muscle determinant. This gene is *macho-1*. The authors found

macho-1 messenger RNA in the myoplasm during early ascidian development. When they depleted eggs of *macho-1* mRNA, the resulting embryos were well organized, but lacked 70% of their muscles. Why not 100%? The 42 muscle cells in the ascidian tadpole belong to two distinct lineages, primary and secondary. The myoplasm controls only the development of the 28 cells of the primary lineage. So the 30% of muscle cells that remained in the embryos depleted of *macho-1* mRNA probably correspond to the secondary lineage.

Nishida and Sawada confirmed that depleting eggs of *macho-1* mRNA hampered differentiation of the primary lineage by isolating blastomere B4.1 from such eggs and showing that it could not, as usual, develop into primary muscle. This defect could be rescued by injecting synthetic *macho-1* mRNA into the eggs. Furthermore, overexpression of *macho-1* mRNA in normal embryos led to the production of muscle cells from blastomeres normally fated to give rise to other tissues.

But these experiments do not give any indication of whether *macho-1* is the muscle determinant in myoplasm, that is, whether it lies at the start of the cascade of signalling molecules that must underlie muscle determination. In frogs, β -catenin is a maternally derived protein that is important for the formation of the ventral-to-dorsal polarity of an embryo. Embryos depleted of maternal β -catenin mRNA do not form dorsal structures⁸. Yet if dorsal cytoplasm from such embryos is transferred to the ventral side of normal embryos, it still does induce dorsalization. This indicates that the dorsal determinant is still present

Daedalus

David Jones

David Jones, author of the Daedalus column, is indisposed.

in embryos lacking β -catenin⁹. Thus, β -catenin is important in dorsalization, but cannot start the process.

Macho-1, however, is a true muscle determinant. Nishida and Sawada transferred the myoplasm from eggs lacking *macho-1* mRNA into blastomeres normally fated to give rise to epidermis (the outer layer of the tadpole). This would normally induce muscle formation, but when the myoplasm lacked *macho-1*, it did not. *Macho-1* clearly lies at the head of the muscle-determination cascade.

Which molecules might lie downstream of *macho-1*? The protein encoded by the *macho-1* mRNA is a type of transcription factor — a protein that recognizes certain sequences in DNA and switches on adjacent genes. The effects of *macho-1* on muscle determination are reminiscent of those of the vertebrate transcription factor MyoD. An ascidian relative of MyoD has been identified in the cells that inherit the myoplasm^{10,11}, but it is expressed too late in development for it to be the muscle determinant. Perhaps *macho-1* works to turn on the *MyoD* gene. Whether such a cascade exists in vertebrates will await the identification of a vertebrate *macho-1*.

The ascidians' small genome, small cell number and short life-cycle, together with the development of molecular markers and techniques such as those used here², make them an attractive model system for developmental biologists. These animals could well become as useful an experimental organism as *C. elegans* — and more relevant to chordates such as ourselves.

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Dominant rams lose out by sperm depletion

A waning success in siring counters a ram's high score in competition for ewes.

Male traits such as large body size and weaponry are thought to have evolved to aid males contesting for access to females¹. But in species where females copulate with many males in a single reproductive cycle, sperm also compete in the female's reproductive tract to fertilize ova². Both types of competition occur in the promiscuously mating feral Soay sheep (*Ovis aries*) found on the island group of St Kilda, Scotland^{3,4}. Here we show that constraints on sperm production mean that those males that are most successful in overt contests can become ineffectual in covert sperm competition.

Sperm competition is commonplace in mammals⁵. In feral Soay sheep, females have been recorded copulating with seven or more different males during their two-day oestrus (our unpublished results). The importance of sperm competition in this population is shown by paternity analyses revealing that 74% of Soay twins are sired by different males⁴. Soay males copulate up to 13 times a day³ and have a very high testes:body mass ratio⁶ — both adaptations for sperm competition⁷.

Soay rams compete fiercely for access to females in oestrus, butting the flanks of opponents (seen in 29% of 722 focal watches) or clashing head-on (15% of watches). Examination of skeletons has revealed that some 60% of Soay rams suffer fractures of the cervical vertebrae, presumably as a result of these fights⁸.

Moreover, analysis of the determinants of male copulation rate over the rut (and hence ability to gain access to receptive females) indicates the advantage of greater skeletal and horn size (Table 1, overleaf). Males with large bodies and horns copulated more than smaller competitors as the rut progressed (Fig. 1a; hindleg length:week of rut, Table 1). Although horns and other weaponry are frequently proposed as targets of sexual selection¹, rarely, if ever, have they been shown to increase reproductive success in mammals irrespective of age and body size. In our analysis, however, we control for body size and discount the effect of age *per se*, which is not significant ($P > 0.2$).

The behavioural success of larger males translates into greater reproductive success (more lambs sired; Table 1). But as the rutting season progressed and their copulation frequency increased, there was a decline in the proportion of lambs they sired (Table 1, hindleg length:week of lambing; Fig. 1b). This mismatch between copulation rate and siring success is difficult to explain in terms other than of sperm depletion.

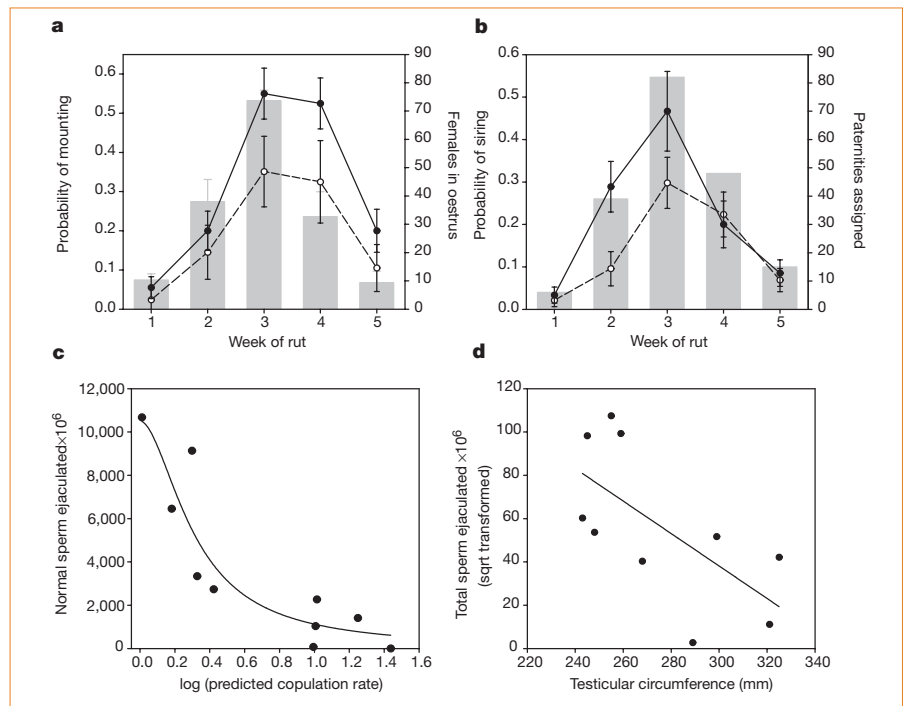


Figure 1 Evidence for sperm depletion in Soay sheep rams. **a**, Temporal variation in the mean probability ($\pm$ s.e.) of being observed mounting during a watch in each week for males with above- (●) and below-average (○) hindleg lengths in each year. Bars, mean number of females in oestrus that week ($\pm$ s.e.). **b**, Temporal variation in the mean probability ($\pm$ s.e.) of being assigned a paternity in a given week for males with above- (●) and below-average (○) hindleg lengths in each year. Bars indicate the total number of paternities assigned to lambs conceived during that week. **c**, Number of normal sperm ejaculated by rams in relation to their predicted copulation rate. Copulation rates were predicted from the male's horn and hindleg length using parameter estimates from the generalized linear mixed model (Table 1); this provides an index of overt competitive ability. Fitted logistic curve: $10508.9/[1 + (\log x/\log_{0.3128})^{1.8235}]$, where x is the copulation rate; $F = 16.475$, $n = 10$, $P = 0.0023$, adjusted $r^2 = 77.5\%$. **d**, Total number of sperm ejaculated (square-root transformed) in relation to testicular circumference. Linear regression: $262.9 - (0.8 \times \text{testicular circumference})$, $F = 5.858$, $n = 10$, $P < 0.05$, adjusted $r^2 = 35.1\%$. Single ejaculates were collected from natural matings of Soay rams using an intra-vaginal device¹⁰. Collections were made between 20 November 1999 and 26 November 1999, in the last two weeks of the rut. Collections prior to this were not logistically feasible.

At its simplest, sperm competition functions as a lottery: the more tickets bought (the more sperm inseminated) the greater the chance of 'winning', in this case gaining a paternity⁷. This mechanism is thought to have selected for the high copulation rates^{5,7} and large testes⁷, as found in mammal species in which sperm competition occurs. The high copulation rates and greater success of large males could result in a widening gap in the sperm that large and small males have available for ejaculates⁷.

Evidence for constrained sperm production comes from domestic sheep^{9,10}. Merino rams that were electro-ejaculated 8 times per day for 18 days showed a decline in sperm numbers per ejaculate of over 85% (ref. 9). We believe that large Soay rams copulating up to 13 times per day³ become increasingly depleted of sperm as the mating season progresses. This results in the sperm in their ejaculates being outnumbered during sperm competition by those

of behaviourally subordinate males with relatively intact sperm reserves, explaining the fall in siring success of larger males.

Ejaculates collected during the later stages of the rut showed that large, frequently copulating males transferred fewer sperm per ejaculate (Fig. 1c), a greater proportion of which were abnormal (Spearman rank correlation between numbers of sperm ejaculated and per cent of abnormal sperm was -0.770 ; $t = -3.410$, $n = 10$, $P = 0.01$). One of the 10 ejaculates contained under 60×10^6 sperm; in domestic sheep, conception rates drop by more than 60% below this threshold¹¹.

It could be argued that the lower siring success of large males towards the end of the rut is due to differential investment in sperm production², with larger males always producing fewer sperm, rather than to a depletion of sperm reserves, but we believe this is highly unlikely. In mammals, there is widespread evidence for testicular size

Table 1 Copulatory and siring success of Soay sheep rams

Term	d.f.	Effect	s.e.	Wald statistic (χ^2)	P
Male copulatory success*					
Watch duration (logged)	1	1.454	0.483	9.07	0.003
No. of females in oestrus	1	0.506	0.117	18.71	<0.001
No. of females in oestrus ²	1	-0.0167	0.00562	8.82	0.003
Week of rut	1	-10.31	3.991	6.68	0.010
Hindleg length	1	-0.0844	0.0650	1.68	0.194
Horn length	1	0.00611	0.00228	7.18	0.007
Hindleg length: week of rut	1	0.0559	0.0207	7.29	0.007
Male siring success†					
No. of females lambing	1	0.0556	0.00810	47.01	<0.001
No. of females lambing ²	1	-0.000293	0.0000725	16.32	<0.001
Week of lambing	1	5.137	1.667	9.49	0.002
Hindleg length	1	0.119	0.0331	12.83	<0.001
Horn length	1	0.00767	0.00168	20.85	<0.001
Hindleg length: week of lambing	1	-0.0258	0.00865	8.85	0.003

We analysed temporal behavioural and paternity data using generalized linear mixed models with a logit link function. These control for repeated measures of behaviour and paternity by fitting male identity and year as random effects¹². A colon between terms denotes two-way interactions.

*Minimal model of male copulatory success. The response is binary, indicating whether a mounting attempt occurred during a focal watch performed in 1996–98; $n=371$. Median watch duration was 1 h.

†Minimal model of male siring success. The response variable takes the form of binomial proportions, being the number of days in a given week on which a male gains a paternity, $n=897$. Includes analysis of 170 paternities assigned between 1988 and 1999⁹. Temporal behavioural and paternity analyses can be compared owing to the correspondence between conception and lambing distributions, gestation being 151.2 ± 1.3 days (mean $\pm$ s.d.)¹². Hindleg length is a linear indicator of skeletal size. Age, weight and basal-horn circumference were tested in each model but were excluded (all $P>0.1$). For further details, see <http://www.ecology.mer.stir.ac.uk/kilda/depletion>.

determining sperm production rate⁷, yet we find a negative correlation between testicular circumference and number of sperm ejaculated (Fig. 1d). This suggests that males producing smaller ejaculates towards the end of the rut had greater capacity for ejaculate production at the start of it. To our knowledge, this is the first demonstration that limitations on ejaculate production may constrain male reproductive success in a wild vertebrate population.

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Biotechnology

Transgenic crops in natural habitats

Although improved crop yields can be engineered by genetically modifying plants, there is ecological concern over whether these plants are likely to persist in the wild in the event of dispersal from their cultivated habitat. Here we present the results of a long-term study of the performance of transgenic crops in natural habitats. Four different crops (oilseed rape, potato, maize and sugar beet) were grown in 12

different habitats and monitored over a period of 10 years. In no case were the genetically modified plants found to be more invasive or more persistent than their conventional counterparts.

In the late 1980s, there were three conjectural risks associated with genetically modified (GM) crops: that they would become weeds of agriculture or invasive of natural habitats; that the introduced genes would be transferred by pollen to wild relatives, whose hybrid offspring would then become more weedy or invasive; or that GM plants would be a direct hazard to humans, domestic livestock or beneficial

wild organisms, for example by being toxic or allergenic. Our study assesses the grounds for the first two of these fears.

We have shown previously that GM oilseed-rape plants did not perform better or persist for longer than conventional plants over a 3-year period¹. We now compare the results of monitoring conventional and GM lines of four crop species over 10 years^{2,3} in a field experiment conducted in 12 different habitats (four in each of Cornwall, Sutherland and Berkshire in the United Kingdom) and repeated in the years 1990, 1991 and 1992 in order to determine whether GM crops would be more invasive or more persistent in natural habitats than non-GM crops. We tested all the crop species and GM constructs that were available in 1990: oilseed-rape and maize plants expressing tolerance of the herbicide glufosinate, sugar beet tolerant of glyphosate ('Roundup'), and two types of GM potato expressing either the insecticidal *Bt* toxin or pea lectin.

The sites were monitored each year to follow the fate of sown individuals, to measure recruitment onto unsown areas nearby, and to determine whether there was any resurgence following natural disturbance in later years. Figure 1 shows the fraction of seeds sown (or tubers planted) that produced mature plants at the end of the first growing season. We found that there were no significant differences in average recruitment between conventional and GM plants for any of the four crops (full details and significance tests are provided in Supplementary Information). None of the crops, GM or conventional, increased in abundance at any of the sites.

Population sizes of all crops declined after the first year as a result of increased competition from native perennial plants. In no case did the GM lines persist for significantly longer than their conventional counterparts. For oilseed rape, seedling establishment was significantly lower for GM plants compared with conventional lines in six out of 12 cases, and were not significantly greater in any case. Subsequent survival was significantly lower for GM lines in three out of 12 cases and significantly higher in two cases. For maize, seedling establishment was significantly lower for the GM line in two out of 12 cases, and significantly greater in no case. Subsequent survival was significantly higher for the GM line in one case, but all lines were extinct by the beginning of the second year.

For potato, survival of planted tubers to the end of the first growing season was significantly lower for GM lines in one case out of eight, and significantly higher in one instance. The few cases of increased GM-plant survival that were significant in the short term did not translate into long-term differences in persistence (see Supplementary Information). Survival of perennating

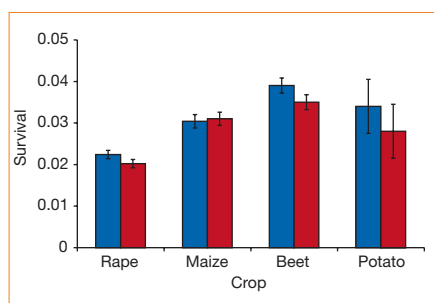


Figure 1 The performance of conventional (blue) and transgenic (red) crops in natural habitats. Survival is the fraction of seeds sown (or tubers planted in the case of potato) that produce mature plants at the end of the first growing season. Error bars, 1 s.e. Data are averaged over habitats and replicates within habitat. In no case did populations of either conventional or transgenic plants increase, and transgenic plants never persisted significantly longer than conventional plants. All populations of maize, rape and sugar beet were extinct at all sites within 4 years of sowing. Potato still survives at one site, 10 years after planting, but the survivors are all conventional.

potatoes was significantly lower for GM lines in one case out of eight, and never significantly higher.

For sugar beet, the genetic background (inbred or outbred) was much more important than whether the plants were genetically modified; outbred lines outperformed inbred lines in three cases out of four. GM lines showed significantly lower recruitment from seed in two cases out of four, and significantly higher recruitment in no case. Survival of GM sugar-beet seedlings was significantly lower for one transition at one site, but all sugar-beet lines were extinct within two years at all four sites. Survival of sea beet was significantly higher than sugar beet at all four sites, but it too was extinct at all sites by the end of the third year. The survival of sea beet on open ground elsewhere in Silwood Park, where potted plants had stood in 1992, sounds the cautionary note that perennial plants can persist for extended periods in extremely odd places.

These experiments involved GM traits (resistance to herbicides or insects) that were not expected to increase plant fitness in natural habitats. Our results do not mean that other genetic modifications could not increase weediness or invasiveness of crop plants, but they do indicate that arable crops are unlikely to survive for long outside cultivation. The ecological impact of plants with GM traits such as drought tolerance or pest resistance that might be expected to enhance performance under field conditions will need to be assessed experimentally when such plants are developed.

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Supplementary information is available at <http://www.nature.com> or as paper copy from the London editorial office of *Nature*.

Materials science

Nanoscale control of chain polymerization

Polymer industries depend on the spontaneous polymerization of molecules into chains in response to an appropriate trigger. We have succeeded in inducing and guiding chain polymerization over tiny distances, initiating and terminating linear propagation at any chosen point to a spatial precision of about 1 nm by using the probe tip of a scanning tunnelling microscope. Being able to exert such fine control over nanoscale fabrication and interconnection should help to take molecular nanoelectronics beyond the present silicon-based-device technology.

We chose a self-ordered monomolecular layer of a diacetylene compound (10,12-nonacosadiynoic acid) adsorbed on a graphite surface because this molecular species polymerizes in a three-dimensional solid state^{1–3} (although this does not guarantee that polymerization can occur in a two-dimensional monomolecular layer). Also, polymerization results in polydiacetylene compounds that behave as conjugated linear polymers and should function as electrically conductive ‘nanowires’ upon

transfer of charge from the surroundings^{4,5}.

Figure 1a shows a typical scanning tunnelling microscope (STM) image of the monomolecular layer. Each bright line running from top to bottom corresponds to a linear array of diacetylene moieties of the self-ordered molecules (the two different alternating spacings between the bright lines are due to the antiphase arrangement of the molecules). We were able to induce chain polymerization in the monomolecular layer by local stimulation with the STM tip (Y.O. and M.A., manuscript in preparation) and to control it efficiently at the nanoscale level (Fig. 1b–e).

First, we created an artificial defect in the form of a 6-nm-wide hole at a predetermined position in the monomolecular layer⁶ (Fig. 1b) by placing the STM tip at this position and applying a positively pulsed sample bias (+5 V in height, 10 μs in width; see also Fig. 1f, g). We again imaged the area shown in Fig. 1b, this time scanning from top to bottom (Fig. 1c) with a negatively pulsed sample bias (–4 V, 5 μs) applied when the scanning tip passed the point indicated by arrow (1), which caused a bright line to appear between the point and the artificial defect. This line represents a polymerized polydiacetylene nanowire, as confirmed by structural analysis, starting at the point indicated by arrow (1) and ending at the defect (Fig. 1h, i).

We imaged the same area once more (Fig. 1d), applying another negatively pulsed sample bias when the tip passed the point indicated by arrow (2): chain polymerization started at this point as well and also terminated at the hole (compare Fig.

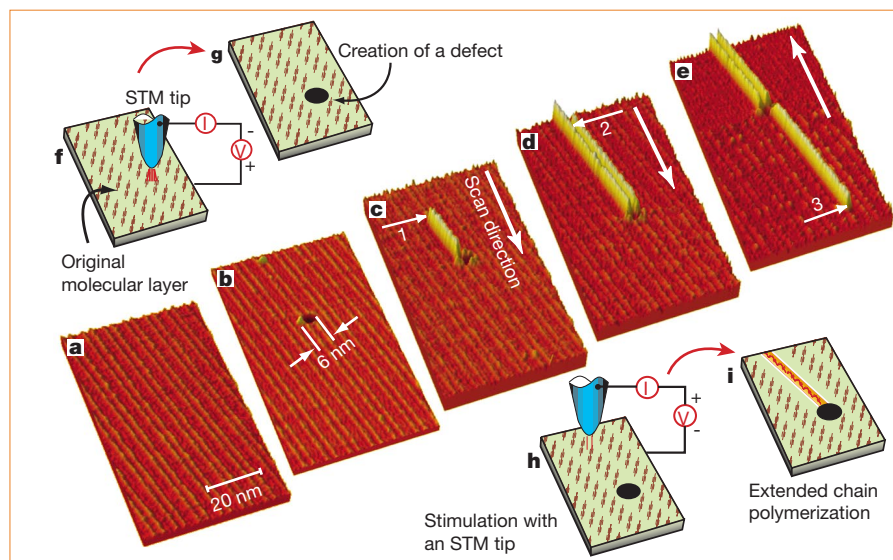


Figure 1 Scanning tunnelling microscope (STM) images and diagrams showing the process of controlling the initiation and termination of linear chain polymerization with an STM tip. STM images were obtained in air at room temperature in constant-current mode. **a**, STM image of the original monomolecular layer of 10,12-nonacosadiynoic acid. **b**, Creation of an artificial defect in the monomolecular layer using an STM tip. **c**, First chain polymerization, initiated at the point indicated by arrow (1) using an STM tip, and terminated at the artificial defect. **d**, Second chain polymerization, initiated at arrow (2). **e**, Third chain polymerization, initiated at arrow (3). **f**, **g**, Creation of an artificial defect in advance with an STM tip. **h**, **i**, Initiation of chain polymerization with an STM tip, and termination of the polymerization at the artificial defect.

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1d with Fig. 1c to see that chain polymerization initiated at arrow (1) typically propagates on both sides of the point of stimulation). We created another polydiacetylene nanowire by initiating chain polymerization at the point indicated by arrow (3) in Fig. 1e.

Our technique should be useful for interconnecting nanostructures, an essential feature of nanotechnology — Fig. 1a–e shows that at least three nanowires can be connected to an object as small as 6 nm, enabling three electrodes (source, drain and gate) of a single-electron transistor, for example, to be wired up even if it is nanometre-sized. The new method will open up possibilities for the fabrication of novel molecular nanoelectronic devices, as well as encouraging the design of various new types of physical and chemical experiments on the nanoscale. They include the measurement of electrical conduction of structurally perfect one-dimensional matter and the analysis of the mechanism of propagation of chain polymerization.

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Gene expression

View of a mouse clock gene ticking

Circadian clocks consist of an ingenious autoregulatory feedback loop whereby the cyclically expressed products of the clock gene are able to inhibit their own expression¹. Here we follow the rhythmic expression of the clock gene *mPer1* in the brain of a living mouse. This model system enables real-time gene expression to be monitored in the intact brain under physiological conditions.

We have previously produced transgenic mice carrying a luciferase (*luc*) reporter gene under the control of the promoter of the oscillating clock gene *mPer1* (refs 2, 3). Using a two-dimensional photon-counting camera, we were able to detect a day–night variation in luciferase-mediated bioluminescence in the suprachiasmatic nucleus (SCN)³, the mammalian brain's circadian

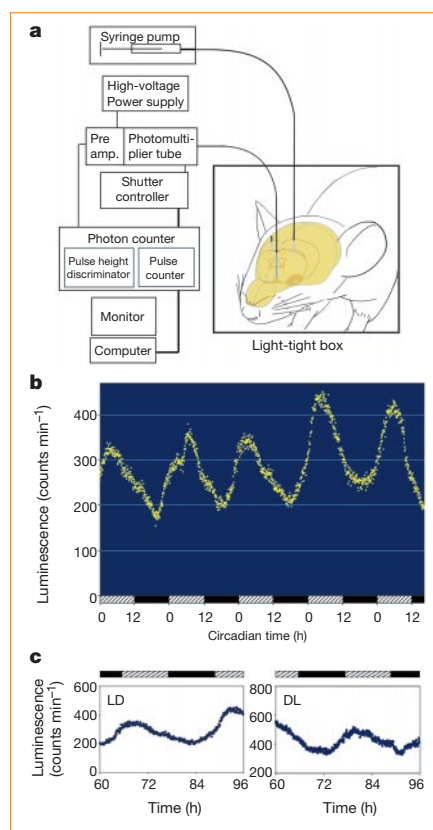


Figure 1 *In vivo* monitoring of bioluminescence from the suprachiasmatic nucleus (SCN). **a**, Experimental set-up. Bioluminescence is detected under constant darkness conditions by means of an optical fibre inserted above the SCN and connected to a photon-counting apparatus with a photomultiplier tube. Luciferin solution (10 mM in artificial cerebrospinal fluid) is infused continuously at 15 μ l h^{-1} by a syringe pump. **b**, Circadian fluctuation of luminescence in the SCN of a representative *mPer1-luc* transgenic mouse previously housed under a 12-h light/12-h dark cycle. Each dot represents the average of luminescence counts collected over 5 min. Hatched and black bars along the x-axis represent subjective day and subjective night, respectively. **c**, Comparison of the phase of luminescence recorded from left, LD-entrained, and right, DL-entrained mice (see text for details of terminology). Time indicated is time since the start of monitoring. Bars and dots as in **b**.

centre⁴, in fresh brain slices from these *mPer1-luc* mice.

We inserted a polymer optical fibre (500 μ m in diameter; 0.5 numerical aperture) just above the SCN of a *mPer1-luc* transgenic mouse using a stereotaxic frame; the other end of the optical fibre was connected to a photomultiplier tube operating as part of a photon-counting apparatus (Fig. 1a). As luciferase requires a substrate for light emission, we continuously infused luciferin dissolved in artificial cerebrospinal fluid through a lateral ventricle. The substrate concentration in the cerebrospinal fluid, sampled from the contralateral ventricle, reached a plateau at about 1.3 mM within 3 hours and remained more or less constant.

With this system, we continuously recorded light emission from the SCN of the transgenic mice *in vivo* under constant darkness. The luminescence showed a clear

circadian fluctuation (Fig. 1b), with a 1.5- to 2.5-fold amplitude and peaks and troughs at circadian time (CT) 4–6 and CT15–20, respectively (where CT0 is subjective dawn and CT12 is subjective dusk).

This temporal profile of bioluminescence strikingly resembles the expression profile of native *mPer1* messenger RNA. The phase lag between transcription of luciferase mRNA and the production of luminescence was only 0–2 h. The circadian period calculated from the intervals between bioluminescence peaks was about 24 h, which is close to the period of locomotor activity of this transgenic mouse line (24.0 ± 0.05 h; $n = 7$).

To confirm that this fluctuation in luminescence represents an endogenous oscillation, we recorded the luminescence from two groups of mice housed for at least two weeks under alternating 12-hour light–dark cycles, with lights on at either 7:00 h (LD-entrained mice) or 19:00 h (DL-entrained mice). Both groups were hooked up to the monitoring system at 14:00 h and the luminescence emitted was recorded under constant darkness. As expected, the luminescence recorded from LD-entrained mice oscillated in the opposite phase to that of DL-entrained animals (Fig. 1c).

Capturing the effect from a luciferase reporter transgene with optical-fibre-mediated photon-counting technology offers a way to measure real-time gene expression profiles in the intact brain under physiological conditions. To our knowledge, this is the first time that a ticking clock gene has been monitored in a living mammal. Real-time optical imaging of gene expression, in combination with real-time measurement of electrophysiological, metabolic and behavioural parameters, should advance the study of mammalian brain function.

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Functional annotation of a full-length mouse cDNA collection

The RIKEN Genome Exploration Research Group Phase II Team and the FANTOM Consortium*

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The RIKEN Mouse Gene Encyclopaedia Project, a systematic approach to determining the full coding potential of the mouse genome, involves collection and sequencing of full-length complementary DNAs and physical mapping of the corresponding genes to the mouse genome. We organized an international functional annotation meeting (FANTOM) to annotate the first 21,076 cDNAs to be analysed in this project. Here we describe the first RIKEN clone collection, which is one of the largest described for any organism. Analysis of these cDNAs extends known gene families and identifies new ones.

In mammals and higher plants, interpreting the genome sequence is not straightforward: coding regions are interspersed with noncoding DNA, and an individual gene may give rise to many gene products. Thus, genomic sequence cannot be reliably decoded to identify the spectrum of messenger RNAs (the transcriptome) and their corresponding protein products (the proteome). This problem is illustrated by the different estimates of the number of human genes (30,000, 35,000 and 120,000)^{1–3}. Although gene prediction programs have become more accurate and sensitive, the sequence of a full-length cDNA clone provides more reliable evidence for the existence and structure of a gene. The Mouse Gene Encyclopaedia Project aims to identify and sequence every transcript encoded by the mouse genome. Here, we report the characterization of our first cDNA set of 21,076 mouse clones (some of which are derived from the same transcripts).

Strategies

In the first phase of the project, we prepared around 160 full-length enriched^{4,5}, normalized and subtracted⁶ cDNA libraries from various tissues and developmental stages. From these, we collected and clustered 930,000 3' end sequences to produce about 128,600 groups that were targeted for sequencing.

In the second phase of the project, we selected a single clone from each cluster for sequencing. Preference was given to clones from libraries estimated to contain the highest representation of full-length transcripts. To expedite sequencing, we focused on relatively short cDNAs (Fig. 1), which are probably biased in favour of 5' truncated clones. To increase the likelihood of discovering new genes, we also biased our selection towards clones with novel 3' end sequences. We sequenced 21,076 cDNA clones, with average length 1,257 base pairs (bp); the longest clone sequenced was 6,327 bp (see Supplementary Information Fig. 1A). All sequences have been registered in the public sequence database DDBJ, except for 1,908 cDNAs assembled using sequences from public expressed sequence tag (EST) databases (available at <http://genome.gsc.riken.go.jp/genome/fantom/viewer/est/>). We estimated using the PHRED base-calling program^{7,8} that the average accuracy of our sequences was 99.1%; 72% (15,236 clones) of clones showed > 99% accuracy and 6,739 sequences (32%) were determined at > 99.9% accuracy (see Supplementary Information Fig. 1B).

We extracted the open reading frame (ORF) of each full-length sequence using the RIKEN DECODER program (see Methods). DECODER corrected frame-shifts in 3,376 (15%) of 21,076 clones. The likelihood that the sequence selected was correct is given by a score (Va) calculated in light of the Kozak consensus, preferred codon usage and position of the initiation codon. The probability that a frame shift occurred was determined using quality values (PHRED scores).

Annotation of cDNAs

An international meeting was held to facilitate functional annotation of the cDNA sequences. Participants contributed to the development of a web-based annotation interface that should expedite future annotation of additional clones in the Mouse Gene Encyclopaedia project. We agreed on annotation vocabularies and the application of Gene Ontology (GO) terms (<http://genome.gsc.riken.go.jp/FANTOM/>). Before the FANTOM meeting, a set of RIKEN clones with significant similarity to mouse genes represented in the databases of Mouse Genome Informatics (MGI) was annotated; 4,248 RIKEN clones were found to be identical by human curation to mouse genes in MGI (referred to as the MGI-confirmed set).

There was significant redundancy in the cDNA set. Duplication may have resulted from a number of factors including mistakes made when samples were regrided, internal initiation of reverse transcription, incomplete or variable splicing and differences in polyadenylation site usage, which may account for about 19% of true 3' end variability⁹. To cluster redundant clones, we compared all the sequences pairwise using FLAST, a sequence comparison program based on DDS¹⁰, and grouped them on the basis of sequence similarity. To assess cluster fidelity, sequences were assembled using CAP3¹¹ and aligned using CLUSTALW¹², and visually inspected. This placed 8,207 clones into 2,957 clusters, reducing the size of the cDNA clone set to 15,826 unique genes and the MGI-confirmed set to 2,921 unique genes. Further analysis of RIKEN clones in the MGI-confirmed set revealed some instances where non-overlapping clones could be added to existing clusters or grouped together on the basis of curatorial association with the same MGI gene. Therefore, the actual number of genes in the MGI set was reduced from 2,921 to 2,390, and the number of genes represented by the whole RIKEN set was reduced to 15,295. This is an overestimate of the total gene number in the RIKEN set, as we expect a similar compression to occur for clones outside the MGI set with further cluster-orientated analyses that consider external data sets. On the basis of the observed redundancy in the MGI set (roughly 20%), we have estimated the number of genes in the non-MGI set to be at least 10,500. Therefore, there should around 12,890 unique genes in the complete collection.

Redundant clones were not eliminated from the RIKEN database, because many clusters contain genuine alternate transcripts from single genes (see below). All nonredundant clones were annotated; for clusters, a single sequence was annotated and the annotation extended to other clones within a given cluster. The number of genes in each category ('MGI-confirmed', 'identical to', 'similar to' and so on) is shown in Table 1.

We functionally classified cDNAs by assigning GO terms¹³ (see Methods). We assigned one or more GO terms to 3,025 of the

9,902 RIKEN clones with definitive coding potential (Table 2). The putative functions of the clones are well distributed among the major categories. (<http://www.gsc.riken.go.jp/genome/fantom/viewer/>).

Analysis of length of cDNAs

Three approaches were used to assess to what extent the clones in the RIKEN cDNA collection were 'full-length'. (1) Clones in the MGI-confirmed set were compared with other published cDNAs for the same genes containing complete coding sequences (CDS), to determine whether the clones span the entire coding sequence; (2) the fraction of computationally predicted CDSs among all clones was calculated; (3) and the fraction of clones annotated as full-length by curators was determined. These analyses gave reasonably similar estimates of the percentage of full-length clones in the

collection: 63%, 53% and 59%, respectively. As the sizes of clone inserts in the categories designated 'motif-containing proteins' and 'hypothetical proteins' were similar to the sizes of clone inserts in the 'MGI-confirmed', 'identical to', 'homologue to', 'similar to' and 'related to' categories (see Supplementary Information Table 2A), we believe that 60–70% of the cDNAs encoding motif-containing and hypothetical proteins (potentially the most novel ones in the RIKEN set) will also be full-length. This validates the cap-trapping method and shows that our clones will be a valuable resource for studying transcription regulatory elements in the 5' untranslated regions (UTRs; see Supplementary Information Table 5B).

A significant number of RIKEN cDNAs are shorter than published cDNAs encoding known genes (see Supplementary Information Table 2A). Some are likely to be truncated and unspliced forms, although alternative transcripts generated from genuine functional promoters, and transcripts generated from cryptic internal promoters, may be other sources of such clones.

Alternative splicing leading to exon skipping, extension, deletion or truncation increases the complexity of gene expression products and the proteome. One study¹⁴ indicated that 22% of human genes may be alternatively spliced. An EST-based analysis of 475 disease-associated genes suggested that one in three genes exhibits alternative splicing¹⁵. This is also evident in the RIKEN cDNA set, of which 220 display potential alternative splicing (see Supplementary Information Table 2B). Furthermore, about 6% of the MGI clones are probably splice variants of known genes.

Ninety-five clones (1.2%) were in the reverse orientation. We confirmed that the inserts were probably inserted into the vector correctly; whether these clones represent antisense transcripts and are important physiologically remains to be determined.

cDNAs representing metabolic enzymes

Over 100 clones representing newly identified genes in mouse were assigned to various metabolic pathways. This was achieved by converting the GO numbers assigned to clones to EC numbers that designate enzymes (see <https://genome.gsc.riken.go.jp/genome/fantom/bono/pathway/index.cgi?org=mmu>).

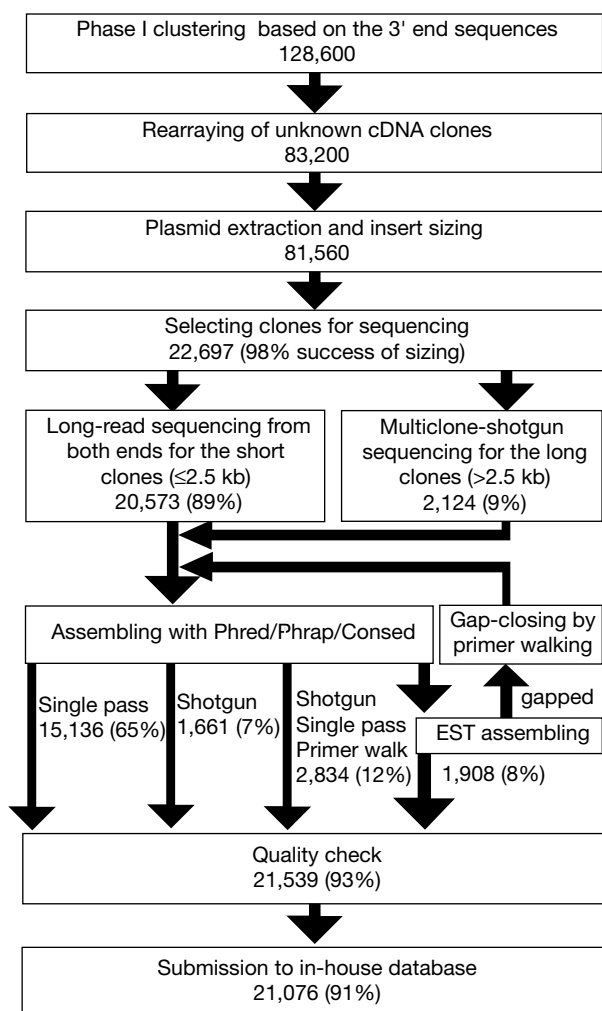


Figure 1 Phase II full-insert sequencing flow chart. We listed 83,200 clones for full-insert sequencing from 128,600 Phase I clusters. To expedite sequencing and increase the likelihood of discovering new genes, we focused on short cDNAs with novel 3' end sequences (22,697 clones). Clones shorter than 2.5 kilobases (kb) were sequenced from both ends using long-read Licor DNA4200 sequencers; clones longer than 2.5 kb were subjected to multi-clone shotgun sequencing (M. Yoshino *et al.*, unpublished) using RISA (RIKEN Integrated Sequence Analysis)^{23,24}. Electropherograms from the four sequencers used (RISA (Shimadzu), Licor DNA 4200 (Licor), ABI377 and ABI3700 (Applied Biosystems Inc.)) were base-called by PHRED, assembled by PHRAP and edited with CONSED^{7,8,25} in three steps. The computer-assisted system assembled the raw sequence data (15,136 and 1,661 clones, respectively, from Licor and Shotgun strategies); we closed the gaps (1,908 assemblies) using public EST databases; and remaining gaps were resequenced by primer walking (2,834 assemblies).

Table 1 Gene categories

Gene categories	Genes	Clones
MGI-confirmed	2,390	4,248
Identical to	488	841
Similar to	703	930
Homologue to	3,550	5,525
Related to	564	841
Motif-containing protein	573	740
Hypothetical protein	1,634	2,086
Unclassifiable transcript	3,251	3,626
Unclassifiable	2,142	2,239
Total	15,295	21,076

Number of genes and clones assigned to each gene category. The definition of each gene category is described in the Methods. Here, a gene refers to a representative clone or a singleton.

Table 2 Number of clones assigned to GO functional categories

GO term	GO ID	Number
Enzyme	GO:0003824	943
Nucleic acid binding	GO:0003676	725
Ligand binding or carrier	GO:0005488	687
Structural protein	GO:0005198	459
Signal transduction	GO:0004871	361
Transporter	GO:0005215	332
Motor	GO:0003774	208
Chaperone	GO:0003754	94
Enzyme inhibitor	GO:0004857	75
Cell cycle regulator	GO:0003750	60
Other		68
Total		4,012

Any given sequence may have been assigned to more than one category. We assigned 4,012 GO functional terms to 3,025 genes as described in the text.

Orthologues of human disease genes

Identifying orthologues of human disease genes in model organisms and creating animal models of human disorders should help us to understand the relationships between genetic variants and human diseases, and may be useful for testing diagnostic and therapeutic strategies. To find orthologues of human disease genes in the RIKEN cDNA set, we compared the clones to 288 human disease gene orthologues compiled for the *Drosophila* genome sequence paper¹⁶. Of this list, 118 genes (44%) share significant protein sequence identity with one or more of the RIKEN clones. We found novel mouse orthologues for ten of these human disease genes: two cancer-related genes (DEK oncogene and BCR), three genes related to neurological disorders (dysferlin, MJD and USH2A), three genes related to malformation syndromes (CKN1, PEX1 and Tafazzin), and two genes related to haematological disorders (α -haemoglobin and XK).

Identification of new mouse genes

Some of the cDNAs in the 'similar to', 'homologue to', 'related to', 'motif-containing protein', 'hypothetical protein' and 'unclassifiable transcript' categories are likely to represent new mouse genes. As in other completed genomes¹⁷, many new genes are members of large multigene families associated with cellular differentiation and signal transduction. For example, we identified 251 genes in the non-redundant clone set encoding zinc-finger containing proteins. For many, there was no evident relationship to any other known gene. Ten cDNAs encoded proteins that contain the SAP domain, a DNA-binding motif¹⁸ (see Supplementary Information Fig. 2). Five of the ten mouse proteins containing this domain have not been characterized, including those that match newly identified predicted human genes. An additional 31 RIKEN clones were annotated as homeobox genes, including seven for which this was the only annotated feature.

Seventy-four of the cDNAs showed recognizable homology to known protein phosphatases, or contained a phosphatase motif. Of these, perhaps the most interesting are 14 clones that are predicted to be members of the dual-specificity protein phosphatase family (see Supplementary Information Table 4A). Each is likely to be a new member of this family and, on the basis of the literature, a possible candidate disease gene or tumour suppressor.

One-hundred and ninety-three cDNA clones in the RIKEN set were identified as protein kinases. Most are closely related to known serine-threonine and tyrosine kinases; only 14 were identified solely on the basis of the consensus kinase signature motif (see Supplementary Information Table 4B). Most of these correspond only to the kinase domain of known genes and may therefore represent novel transcripts.

Although the RIKEN set seems to contain many novel cDNAs, it contains relatively few known or new host defence genes. Most such genes are induced by immunological challenges, and lymphoid organs such as spleen contain complex mixtures of cell types in different states of activation. Therefore, even following induction, immune-related transcripts have low abundance in total tissue mRNA. Future efforts in compiling the mouse gene encyclopaedia will include production and sequencing of libraries from various stimulated immune cells.

Determining protein domains and families

For sequences without recognizable homology to a known gene, we searched for functional motifs in an attempt to predict their protein products. We performed FASTA searches against InterPro and HMMER searches against the TIGR-FAM database using DECODER-predicted protein sequences. In addition, we searched the Pfam database with the program ESTwise using the RIKEN clone nucleotide sequences. The search of RIKEN clones against InterPro data is shown in Supplementary Information Table 4C. InterPro motifs were identified in 3,204 new mouse genes.

Whenever possible, InterPro identifiers were used to allocate the GO terms.

New protein motifs

We used maximum density subgraph analysis¹⁹ to identify six new motifs in our cDNAs, which were not present in the Pfam, ProDom and InterPro databases. Hidden Markov models (HMM) were constructed for these motifs and used to search the Swiss-Prot-TrEMBL nonredundant database using HMMER version 2.1.1. Two such searches resulted in the discovery of motifs in the organic-anion transporting polypeptide (Oatp) family sequences (see alignment at OATP, Supplementary Information Fig. 3B). A phylogenetic analysis (see Supplementary Information Fig. 3A) indicates that RIKEN clones 7516, 15434 and 18937 may belong to a new Oatp subfamily. The results of other HMM searches are shown in Supplementary Information Table 5A.

Untranslated regions

Sequences affecting the translation and stability of mRNAs are found in the 5' and 3' UTRs. Using PatSearch²⁰, we screened our clone set for UTR-specific functional motifs in UTRsite (<http://bigarea.area.ba.cnr.it:8000/EmBIT/UTRHome>). This occasionally added to the functional annotation of clones. For example, a histone 3' UTR stem-loop structure in two unclassifiable RIKEN cDNAs (RIKEN clones 10172 and 22851) indicates that these clones correspond to the 3' UTRs of mRNAs encoding histone proteins (see Supplementary Information Table 5B for these and other common motifs in the RIKEN clones).

Chromosomal mapping of cDNA clones

RIKEN clones corresponding to those in the Whitehead Mouse and

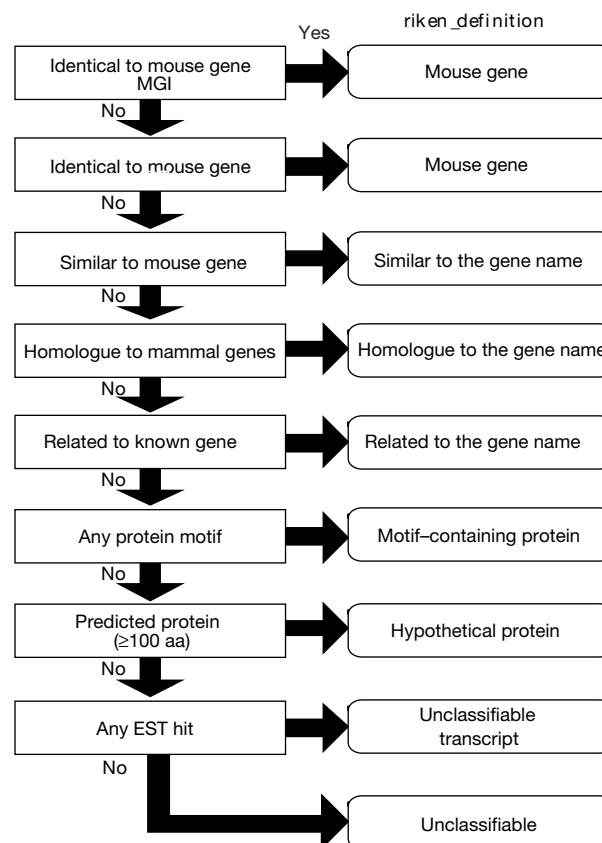


Figure 2 The criteria used in assigning RIKEN definitions (riken_defs). See Methods for details.

Jackson Laboratory radiation hybrid databases were directly mapped onto the mouse genome (see Supplementary Information Fig. 4 and Table 6). We also mapped 12,191 clones to human genomic sequences and then onto the mouse genome on the basis of synteny between the mouse and human genomes (<http://www.gsc.riken.go.jp/e/FANTOM/map/>).

We identified 817 hypothetical transcripts for which there were no corresponding human genes in the RefSeq, human UniGene or Ensembl transcript databases. Of these, 485 mapped to one or more GenScan-predicted exons in the human draft sequence. Among the 485 putative transcripts, 174 perfectly matched the GenScan predictions; 311 showed only partial matches because GenScan seemed not to have predicted one or more exons. The remaining 332 cDNAs did not hit any exon predicted by GenScan. These data strongly support the importance of cDNA sequencing in the identification of genes that would not otherwise be discovered in genomic sequences and indicate the need for caution when using *ab initio* predictions as the primary source for genome annotation.

Discussion

Functional annotation of the first RIKEN mouse cDNA set clearly validates the overall strategy, but also suggests the need for further refinements and for similar projects in other species. At least some unclassifiable transcripts probably represent unprocessed nuclear RNA that could potentially be avoided by isolating cytoplasmic RNA. Future prioritization will include 5' as well as 3' end sequencing. In this clone set, we focused on shorter transcripts and this may have had the unintended consequence of enriching for truncations and abundant gene products already present in the MGI database.

Information about these clones is available at RIKEN (<http://www.gsc.riken.go.jp/e/FANTOM/viewer/>) and Mouse Genome Informatics (<http://www.informatics.jax.org> and mirror sites). We would welcome suggestions for annotation. Ultimately, however, the annotation of this and other clone sets, as well as the human, mouse, rat and other genomes, will come from careful experimental analysis of the identified coding sequences. A variety of techniques, including microarray analysis and two-hybrid screens, will lend significant experimental support for functional assignments and lead to the discovery of pathways and gene families. As the new mouse genes in this set become better characterized, revised nomenclature and other biological data will be incorporated into their MGI and FANTOM records. Further computational analyses, including cross-species comparisons, will further elucidate the functions of the newly identified genes and may assist in the identification of genomic regulatory regions. □

Methods

Phase I

All cDNA libraries were prepared from C57BL/6J mouse mRNA using a strategy designed to enhance representation of full-length transcripts. About 160 cDNA libraries were enriched in full-length inserts by applying several technologies including cap trapping⁴⁵, thermoactivation of reverse transcriptase by trehalose²¹, normalization and subtraction⁶ and vectors designed for the preferential cloning of long inserts (P. Carninci, manuscript in preparation).

Phase II

The representative clones were regridged. The sequencing strategy (Fig. 1) and sequence editing approaches are described in Supplementary Information methods.

Gene assignments and functional annotation of genes

We used a variety of software programs, including BLASTN, BLASTX, FASTA/FASTY (<ftp://ftp.virginia.edu/pub/fasta/>), DECODER, EST-WISE (<http://www.sanger.ac.uk/Software/Wise2/>) and HMMER (<http://hmmerr.wustl.edu/>), to search databases including NCBI-nr, Locus Link, SwissProt, SwissProt TrEMBL, TIGR nraa, PFAM, TIGR-FAM, UniGene, the TIGR Gene Indices, UTRdb and UTRsite, and a number of species-specific databases (see Supplementary Information Table 7A and B). (DECODER²⁶ is an amino-acid translation program designed to suggest the position of experimental frame-shift errors, and predict amino-acid sequences for full-length cDNA sequences with PHRED

scores. The program generates artificial insertions into and artificial deletions from the low-accuracy base positions of the original sequence, thereby generating many candidate sequences. The validity of the most probable sequence (the likelihood that it represents the actual protein) is evaluated by using a score (Va) that is calculated in light of the Kozak consensus, preferred codon usage and position of the initiation codon.) Additional analyses were performed using the bioSCOUT program (LION Bioscience). Protein domain analyses were conducted at the European Bioinformatics Institute using the InterPro software program.

Curators annotated clones with the help of the FANTOM+ interface, which allowed users to view pre-computed similarity and motif search results, to launch additional searches, and to transfer the annotation from any of these to the FANTOM database.

The aim of the FANTOM meeting was to assign each RIKEN clone a RIKEN definition (riken_def) to indicate its most likely function and/or status on the basis of similarity to known genes. A supplementary RIKEN definition line (riken_def_suppl) was available in the interface for additional annotation. Annotation of RIKEN clones with significant similarity to known sequences was guided by the gene or gene product descriptors of the reference sequences to which the RIKEN clones were similar. In general, the riken_def was derived from the gene descriptor of the reference sequence that had the highest similarity to the RIKEN clone sequence. When the RIKEN clone was highly similar to several genes, an annotation hierarchy was used to choose the riken_def, based on the species of origin and descriptor content for the candidate reference sequences (Fig. 2).

Priority was given to reference sequence descriptors from which functional information could be inferred, even if sequences with less informative descriptors were more similar to the clones. Annotations from highly curated databases (MGI and SwissProt) were preferred and provided convenient entry points into the GO vocabularies. Informative descriptors from mouse genes identical to RIKEN clones were the first choice for annotation. Official gene nomenclature was used preferentially for the 'MGI-confirmed' set. For RIKEN clones identical to mouse genes not represented in MGI ('identical-to') or with non-identical similarity to known genes, riken_defs were derived from informative gene descriptors according to the following species priority: identical mouse > non-identical mouse > non-mouse mammal > non-mammal. The controlled vocabulary prefix terms 'similar to', 'homologue to' and 'related to' were used in the riken_def line to indicate that a gene descriptor was derived from non-identical mouse, non-mouse mammal or non-mammal sources, respectively. RIKEN clones with no significant sequence similarity to known genes were named on the basis of coding potential, protein motif signature and representation in mouse, human or rat EST databases. RIKEN clones with no significant similarity to known sequences, but with predicted protein motifs found in Pfam and/or InterPro, were named 'motif name'-containing protein'. Clones with no known sequence similarity or domain hits, but with coding potential ≥ 100 amino acids and EST representation, were named 'hypothetical protein'. Clones belonging to none of the above groups, but with matches to ESTs, were referred to as 'unclassifiable transcript'. Clones with no EST matches were called 'unclassifiable'. New mouse genes discovered in the RIKEN clone set will be assigned official nomenclature in MGI according to a defined syntax: gene symbol, (Riken Clone Identifier)Rik; gene name, Riken cDNA (Riken Clone Identifier) gene (for example, 2610307C23Rik; Riken cDNA 2610307C23 gene). For novel genes represented by RIKEN clusters, nomenclature will be taken from the clone identifiers of the representative clones for each cluster.

Computational identification of full-length clones

See Supplementary Information Table 2A.

Assignment of gene ontology (GO) terms

See Supplementary Information Table 7.

Mapping RIKEN clones using mouse radiation hybrid (RH) data

Repeat-masked RIKEN clone sequences were BLAST-searched against the Whitehead Mouse RH database and the Jackson Laboratory RH database. Identity $\geq 98\%$ over more than 100 bp was considered an exact correspondence. In total, 8,960 sequences were unique after eliminating redundancy between these two databases. The RIKEN sequences were searched by BLASTN against this nonredundant set. Among the RIKEN clones, 3,398 were matched; 2,469 and 3,085 RIKEN clones were mapped onto the Whitehead Mouse RH database and The Jackson Laboratory RH database, respectively.

Mapping of RIKEN full-length cDNAs onto the human genomic sequence

To detect even short exons (20 bp), we conducted a BLASTN search (E -value = 1.0) between repeat-masked RIKEN clones and the human genome sequences (15 June 2000 version) provided by the Center for Biomolecular Science and Engineering, UCSC, which comprises three billion bases and represents each chromosome in one continuous contig (at the time of analysis 19.7% of the sequence was incomplete, that is, N base). These selected exons were used for mapping our cDNAs. The criterion for mapping was much more stringent, based upon the sum of the lengths of homologues being > 200 bp. All nonredundant cDNAs were compared pairwise to the 10,239 human reference genes of RefSeq, 81,963 UniGene clusters and 37,720 Ensembl transcripts. To identify the candidates for the hypothetical genes, we eliminated the RIKEN cDNAs that showed homology (≥ 100 bp at $> 70\%$ identity by BLASTN) to the above database. For mapping, an average of 85–88% identity was reported between mouse and human mRNAs of orthologous sequences²².

The RIKEN mouse cDNA clones will be publicly available in May 2001 when we have replicated the clones and sent them to the distributor. Information on how to obtain these clones can be obtained from <http://genome.gsc.riken.go.jp>.

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Measurement of stellar age from uranium decay

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The ages of the oldest stars in the Galaxy indicate when star formation began, and provide a minimum age for the Universe. Radioactive dating of meteoritic material¹ and stars² relies on comparing the present abundance ratios of radioactive and stable nuclear species to the theoretically predicted ratios of their production. The radioisotope ²³²Th (half-life 14 Gyr) has been used to date Galactic stars²⁻⁴, but it decays by only a factor of two over the lifetime of the Universe. ²³⁸U (half-life 4.5 Gyr) is in principle a more precise age indicator, but even its strongest spectral line, from singly ionized uranium at a wavelength of 385.957 nm, has previously not been detected in stars⁴⁻⁷. Here we report a measurement of this line in the very metal-poor star CS31082-001⁸, a star which is strongly overabundant in its heavy elements. The derived uranium abundance, $\log(U/H) = -13.7 \pm 0.14 \pm 0.12$ yields an age of 12.5 ± 3 Gyr, though this is still model dependent. The observation of this cosmochronometer gives the most direct age determination of the Galaxy. Also, with improved theoretical and laboratory data, it will provide a highly precise lower limit to the age of the Universe.

We are currently undertaking a large programme of high-resolution spectroscopy—at the ESO Very Large Telescope and UVES⁹ spectrograph—of extremely metal-poor stars selected primarily from the Ca II HK survey of ref. 8. The star CS31082-001 (V-band magnitude $V = 11.7$) has been identified as very metal-poor (T.C.B. *et al.*, manuscript in preparation). One of us (the observer, V.H.) has noted that this star is similar to another well studied very metal-poor star, CS22892-052^{6,10,11}, in that it exhibits a large enhancement (relative to iron) of elements formed by the neutron-capture r-process. High-resolution spectroscopic observations of CS22892-052 had allowed a precise measurement of ²³²Th from the 401.9-nm line, but based on the abundance of this slowly decaying species, the age of this star remained fairly uncertain: 15.6 ± 4.6 Gyr (ref. 4).

Moreover, the newly discovered star CS31082-001 exhibits considerably less contamination of the atomic line spectrum by molecular bands of CH and CN than does CS22892-052. The Th II line at 401.9 nm is also unusually strong and clear of contaminants; in fact, a total of 14 Th II lines are detected, 11 of which were selected for measurement. Only the 401.9-nm line has previously been accurately measured in a stellar spectrum, and 10 additional lines in CS31082-001 appear to be first detections. More importantly, the strongest U II line is clearly detected (Fig. 1). We note that no lines are seen at this wavelength in the stars HD115444⁴ or HD122563, which have atmospheric parameters and iron abundance similar to those of CS31082-001, removing any doubt that the line we see is indeed due to U II. To illustrate the quality of our

data, a one-hour integration at a resolving power of $R = 70,000$ yields typical signal-to-noise (S/N) ratios of 150 at 385 nm, and 250–300 at 650 nm. The region of the U II line is currently covered by eight such individual spectra. Other lines of U II are below 1 mÅ in equivalent width in our spectra, and blended with stronger lines.

Using available visual and infrared photometry and the detailed spectroscopic constraints from our own observations, the best model atmosphere for CS31082-001 has the parameters effective temperature $T_{\text{eff}} = 4,825$ K, gravity $[g(\text{cm s}^{-2})] \log = 1.5 \pm 0.2$, and microturbulence $\xi_{\text{micro}} = 1.8 \pm 0.2 \text{ km s}^{-1}$. Thermal non-equilibrium effects should be unimportant for Th II and U II, as these elements are virtually fully ionized and the observed transitions come from the ground level or from a very low excitation level. We then derive the following abundances (on the scale where $\log \epsilon(H) = 12$): $\log \epsilon(\text{Fe}) = 4.60 \pm 0.06$, $\log \epsilon(\text{Os}) = 0.49 \pm 0.10$; $\log \epsilon(\text{Ir}) = 0.40 \pm 0.12$, $\log \epsilon(\text{Th}) = -0.96 \pm 0.03$, and $\log \epsilon(U) = -1.70 \pm 0.10$. The quoted errors are the standard deviation on the mean abundance from at least three lines when available, otherwise they are estimates from the S/N ratio of the spectrum. They do not include systematic errors on oscillator strengths, which are extremely difficult to assess. For U we recomputed the partition functions of U I, U II and U III from the tables of energy levels in ref. 12, and adopted the oscillator strength from the laser-induced fluorescence measurements of ref. 13.

The iron abundance of CS31082-001 is about 1/800 of that of the Sun, while the heaviest detected stable elements, Os and Ir, are about 1/9 as abundant as in the Sun. CS31082-001 is exceedingly rich in spectral lines from most or all of the heavy elements, and a detailed discussion of the individual elements and the process(es) by which they were formed will be reported elsewhere (R.C. *et al.*, manuscript in preparation).

All the heavy elements in a star as metal-poor as CS31082-001 were likely to have formed during a very short interval early in the history of the Galaxy itself^{14,15}. The abundance ratio, r , between a radioactive and a stable comparison species observed Δt Gyr after production is then $\rho = \rho_0[\exp(-\ln 2 \times \Delta t/t_{1/2})]$, where ρ_0 is the initial production ratio and $t_{1/2}$ is the half-life of the radioactive isotope.

In order to determine an absolute radioactive decay age, the initial production ratio is needed. Such ratios are predicted by theoretical nucleosynthesis models, validated by requiring that the

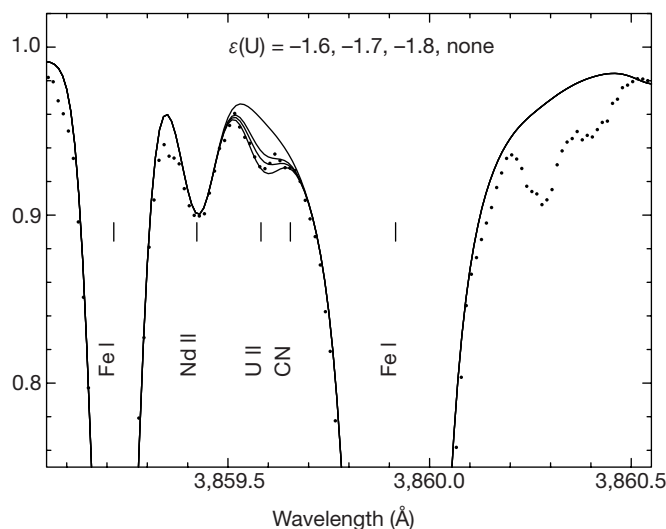


Figure 1 The spectrum of CS31082-001 around the U II line at 385.959 nm. The synthetic spectra (solid lines) were computed with the stellar atmospheric parameters given in the text, and for the three U abundances indicated, adopting an oscillator strength $f = 0.053$ for the line (ref. 13). The observed spectrum (data points) was obtained in four hours, for a total S/N ratio of 300.

predicted abundance ratios for all stable r-process elements in a neighbouring range of mass numbers should be identical to those observed in the star and in the Solar System^{4,16}. The accuracy of a predicted ratio depends both on the adopted nuclear physics model and on the degree to which different production sites yield similar results. The latter has generally been found to be the case for previously studied r-process enhanced stars, but further work is needed to verify it in detail for CS31082–001.

Both theoretical and observational uncertainties should be minimal for elements as close as possible to each other in atomic number. Thus, Os and Ir should be the best stable reference elements in CS31082–001 for the radioactive species ²³²Th and ²³⁸U (any ²³⁵U has decayed to insignificance long ago). From the half-lives of ²³²Th and ²³⁸U (14.05 and 4.468 Gyr, respectively), Δt as a function of the logarithmic decay of Th and U is:

$$\Delta t = 46.7[\log(\text{Th}/r)_0 - \log(\text{Th}/r)_{\text{obs}}] \quad (1)$$

$$\Delta t = 14.8[\log(\text{U}/r)_0 - \log(\text{U}/r)_{\text{obs}}] \quad (2)$$

$$\Delta t = 21.8[\log(\text{U}/\text{Th})_0 - \log(\text{U}/\text{Th})_{\text{obs}}] \quad (3)$$

where Δt is expressed in Gyr, r is a stable third-peak r-process element (here Os or Ir), and the terms such as $(\text{Th}/r)_0$ are the initial production ratios for each pair of species.

Equations (1) and (2) highlight the importance of adding U to the battery of Galactic chronometers: an error of 0.1 dex in a Th abundance propagates as a time equal to the age of the Solar System, neglecting other sources of error. But equation (3) shows that U and Th can be used in concert, with little loss in formal precision but with important overall advantages: the initial production ratio $(\text{U}/\text{Th})_0$ is in principle much less affected¹⁷ by theoretical uncertainties than any of the individual $(\text{U}/r)_0$ because of the proximity of the two elements in their mass numbers. We note that the observational accuracy of (U/Th) is better than for U or Th alone, because errors coming from the choice of model atmosphere parameters largely cancel out.

The observed value of $\log(\text{U}/\text{Th})$ in CS31082–001 is -0.74 ± 0.15 . Here our error estimate includes 0.1 dex reflecting astrophysical observational errors, and 0.12 dex for the uncertainty on the oscillator strength of the U II 385.96-nm line. We only have to introduce an estimate of $\log(\text{U}/\text{Th})_0$ in equation (3) to derive the age of CS31082–001. This is done in Table 1, where we have given the reference used for the value of the production ratio. We have also explored the use of the ratio of U to stable elements. As explained above, it is safest to choose a reference element as heavy as possible (that is, close in mass number to U and Th). Table 1 gives the corresponding results using production ratios for Os and Ir from ref. 4. Any age between 11.1 and 13.9 Gyr is compatible with the various determinations associated with their error bars. We consider the median value 12.5 Gyr as our best present estimate for the age of CS31082–001, with a conservative standard error of 3 Gyr. When increased by 0.1–0.3 Gyr (refs 14, 15), these values give the age of the Galaxy, which is in turn a lower limit to the age of the Universe.

The accuracy of this uranium dating technique is at present limited by incomplete knowledge of a few critical physical data, in particular oscillator strengths and production ratios of the elements produced by the r-process. Further laboratory and theoretical work should enable progress on both these issues, as should the detection of additional stars with enhanced abundances of r-process

elements, similar to CS31082–001. Such work, already in progress, should allow the full potential of the uranium chronometer to be realized. □

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Generic mechanism for generating a liquid–liquid phase transition

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Recent experimental results¹ indicate that phosphorus—a single-component system—can have a high-density liquid (HDL) and a low-density liquid (LDL) phase. A first-order transition between two liquids of different densities² is consistent with experimental data for a variety of materials^{3,4}, including single-component systems such as water^{5–8}, silica⁹ and carbon¹⁰. Molecular dynamics simulations of very specific models for supercooled water^{2,11}, liquid carbon¹² and supercooled silica¹³ predict a LDL–HDL critical point, but a coherent and general interpretation of the LDL–HDL transition is lacking. Here we show that the presence of

Table 1 Ages derived for CS31082–001 as a function of production ratios

Element pair	log (production ratio)	Ref.	log (observed ratio)	Derived age (Gyr)
U/Th	–0.255	4	–0.74 ± 0.15	10.6 ± 3.3
U/Th	–0.10	17	–0.74 ± 0.15	14.0 ± 3.3
U/Os	–1.27	4	–2.19 ± 0.18	13.6 ± 2.7
U/Ir	–1.30	4	–2.10 ± 0.17	11.8 ± 2.5

a LDL and a HDL can be directly related to an interaction potential with an attractive part and two characteristic short-range repulsive distances. This kind of interaction is common to other single-component materials in the liquid state (in particular, liquid metals^{2,14–21}), and such potentials are often used to describe systems that exhibit a density anomaly². However, our results show that the LDL and HDL phases can occur in systems with no density anomaly. Our results therefore present an experimental challenge to uncover a liquid–liquid transition in systems like liquid metals, regardless of the presence of a density anomaly.

Several explanations have been developed to understand the liquid–liquid phase transition. For example, the two-liquid models⁴ assume that liquids at high pressure are a mixture of two liquid phases whose relative concentration depends on external parameters. Other explanations for the liquid–liquid phase transition assume an anisotropic potential^{2,11–13}. Here we shall see that liquid–liquid phase transition phenomena can arise solely from an isotropic pair interaction potential with two characteristic lengths.

For molecular liquid phosphorus P_4 (as for water), a tetrahedral open structure is favoured at low pressures P and low temperatures T , but a denser structure is favoured at high P and high T (refs 1, 6, 8). The existence of these two structures with different densities suggests a pair interaction with two characteristic distances. The first distance can be associated with the hard-core exclusion between two particles and the second distance with a weak repulsion (soft core), which can be overcome at large pressure. Here we will use a generic three-dimensional (3D) model composed of particles interacting through an isotropic soft-core pair potential. Such isotropic potentials can be regarded as resulting from an average over the angular part of more realistic potentials, and are often used as a first approximation to understand the qualitative behaviour of real systems^{2,14–22}. For Ce and Cs, a potential with nearest-neighbour repulsion and a weak long-range attraction was proposed¹⁵. By means of an exact analysis in one dimension (1D), two critical points were found¹⁵, with the high-density critical point interpreted as a solid–solid transition. Then analytic calculations¹⁶, simulations¹⁷ and the 1D exact solution (ref. 18) of the structure factor for a model with a soft-core potential were found to be consistent with experimental structure factors for liquid metals such as Bi. The structure factor for liquid metals was also the focus of a theoretical study of a family of soft-core potentials by mean-spherical approximation¹⁹. More recently, the analysis of the solid phase of a model with a soft-core potential²⁰ was related to the experimental evidence of a liquid–liquid critical point in the K_2Cs metallic alloy²¹. Moreover, for simple metals, soft-core potentials have been derived by first-principle calculations¹⁴ and computed from experimental data². Soft-core potentials are important in the context of supercooled liquids^{23–25}, and the analysis of experimental data for water gives rise to a soft-core potential²⁶.

The isotropic pair potential considered here (Fig. 1a, inset) has a hard-core radius a and a soft-core radius $b > a$. For $a < r < b$ the particles repel each other with energy $U_R > 0$; for $b < r < c$ they attract each other with energy $-U_A < 0$. For $r > c$ the interaction is considered negligible and is approximated to zero. The potential has three free parameters: b/a , c/a and U_R/U_A .

To select the parameters for the molecular dynamics (MD) simulations is not easy, and MD is too time-consuming to study a wide range of parameter values. Hence we first perform an integral equation analysis²⁷, whose predictions we can calculate very rapidly and efficiently. In this technique, one derives the phase diagram by studying the static pair distribution function—which measures the probability of finding a particle at a given distance from a reference particle, and thereby quantifies the correlation between pairs of particles. Mathematically, this function can be written as the sum of an infinite series of many-dimensional integrals, over particle coordinates, involving the pair interaction potential. Because this exact expression is intractable, approximations must be made. Here

we use the hypernetted-chain approximation, which consists of neglecting a specific class of these integrals, leading to a simplified integral equation that can be solved numerically. In the temperature–density (T – ρ) phase diagram, the region where the simplified equation has no solutions is related²⁷ to the region where the system separates into two fluid phases. Thus this technique allows us to estimate the parameter range where two critical points occur, and hence to find useful parameter values for the MD simulations: $b/a = 2.0$, $c/a = 2.2$ and $U_R/U_A = 0.5$. We also use several additional parameter sets in the MD calculations.

Specifically, we perform MD simulations in 3D at constant volume V and number of particles $N = 490$ and 850. We use periodic boundary conditions, a standard collision event list

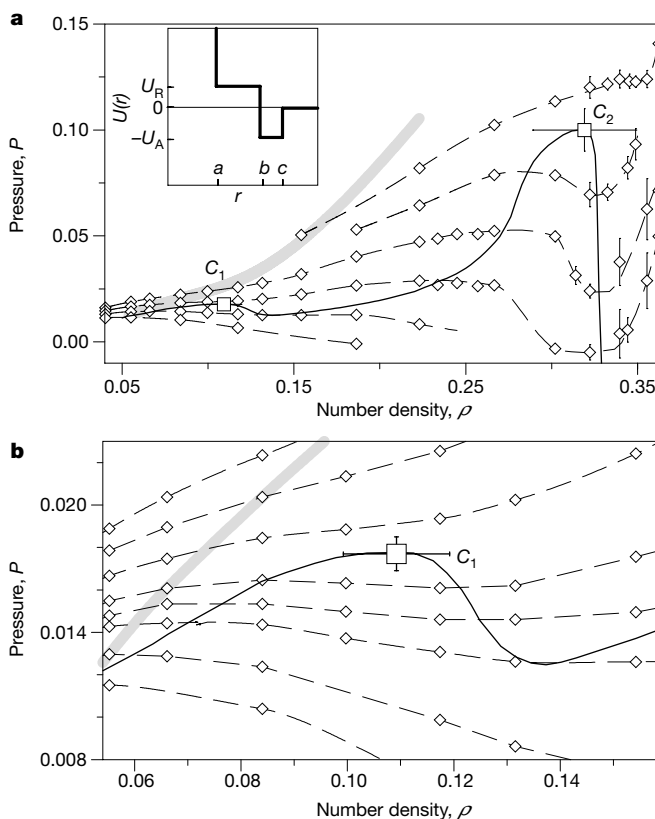


Figure 1 Pressure–density isotherms, crystallization line and spinodal line from the molecular dynamics simulations for the isotropic pair potential in three dimensions. Inset to **a**, the pair potential energy $U(r)$ as a function of the distance r between two particles. Distance a is the hard-core radius, b is the soft-core radius, c is the cut-off radius. Energy U_R is the repulsive energy and U_A is the attractive energy. **a**, Several isotherms (bottom to top) for $T = 0.57, 0.59, 0.61, 0.63, 0.65$ and 0.67 . All quantities are in reduced units: length in units of a , temperature in units of U_R/k_B , pressure in units of U_R/a^3 , (number) density in units of $1/a^3$. Diamonds represent data points and lines are guides for the eyes. The solid line connecting local maxima and minima along the isotherms represents the spinodal line. The two maxima of the spinodal line (squares) represent the two critical points C_1 and C_2 . To determine the crystallization line (grey line)—below which the fluid is metastable with respect to the crystal—we place a crystal seed, prepared at very low T , in contact with the fluid, and check, for each (T, ρ) pair, whether the seed grows or melts after 10^6 MD steps. The spontaneous formation (nucleation) of the crystal is observed, within our simulation times ($\sim 10^5$ MD steps), only for $\rho \geq 0.27$. We use the structure factor $S(Q)$ —the Fourier transform of the density–density correlation function for wave vectors Q —to determine when the nucleation occurs. Indeed, at the onset of nucleation, $S(Q)$ develops large peaks at finite Q ($Q = 12a^{-1}$ and $Q = 6a^{-1}$). For each ρ , we quench the system from a high- T configuration. After a transient time for the fluid equilibration, we compute $P(T, \rho)$, averaging more than 10^5 – 10^6 configurations generated from up to 12 independent quenches, making sure that the calculations are done before nucleation takes place. **b**, Enlarged view of the region around the gas–LDL critical point C_1 for $T = 0.570, 0.580, 0.590, 0.595, 0.600, 0.610, 0.620$ and 0.630 .

algorithm²⁴, and a modified Berendsen method to control T (ref. 28). We find, for each set of parameters, the appearance of two critical points (Fig. 1).

A critical point is revealed by the presence of a region, in the pressure–density (P – ρ) phase diagram, with negative-slope isotherms. In MD simulations this region is related to the coexistence of two phases². The (local) maximum and minimum along an isotherm correspond to the limits of stability of the existence of each single phase (supercooled and superheated phase, respectively). By definition, these maxima and minima are points on the spinodal line for that temperature. Because the spinodal line has a maximum at a critical point, one way to locate a critical point is to find this maximum. In our simulations (Fig. 1), we find two regions with negatively sloped isotherms and the overall shape of the spinodal line has two maxima, showing the presence of two critical points, C_1 and C_2 . Using the Maxwell construction in the P – V plane², we evaluate the coexistence lines of the two fluid phases associated with each critical point (Fig. 2). Considering both the maxima of the spinodal line and the maxima of the coexistence regions in the P – ρ and P – T planes, we estimate the low-density critical point C_1 at $T_1 = 0.606 \pm 0.004$, $P_1 = 0.0177 \pm 0.0008$, $\rho_1 = 0.11 \pm 0.01$ and the high-density critical point C_2 at $T_2 = 0.665 \pm 0.005$, $P_2 = 0.10 \pm 0.01$, $\rho_2 = 0.32 \pm 0.03$. Temperatures are in units of U_A/k_B (where k_B is the Boltzmann constant), pressures in units of U_A/a^3 , and (number) densities in units of $1/a^3$. Critical point C_1 is at the end of the phase-transition line separating the gas phase and the LDL phase, while critical point C_2 is at the end of the phase-transition line separating the gas phase and the HDL phase. Their relative positions resemble the phosphorus phase diagram, except that, in the experiments, C_2 has not been located¹, but is expected at the end of the gas–HDL transition line.

Our phase diagram (Fig. 2) shows the following fluid phases. At high $T > T_2$, the only fluid phase is the gas. At $T_1 < T < T_2$, we find—depending on ρ —the gas alone, or the HDL alone (turquoise

region), or the HDL coexisting with gas (black line in Fig. 2a and green region in Fig. 2c). Below T_1 , the LDL phase appears alone (blue region), or in coexistence with the HDL (orange line in Fig. 2b and orange region in Fig. 2d), or in coexistence with the gas (red line in Fig. 2b and red region in Fig. 2d). The point where the gas–LDL coexistence line merges with the LDL–HDL coexistence line is the triple point. Below the pressure and temperature of the triple point, the LDL is not stable and separates into gas and HDL.

For phosphorus the liquid–liquid transition occurs in the stable fluid regime¹. In contrast, for our model, it occurs in the metastable fluid regime (see Fig. 1). We therefore wish to understand how to enhance the stability of the critical points with respect to the crystal phase. We find that by increasing the attractive well width $(c - b)/a$, both critical temperatures T_1 and T_2 increase, and hence both critical points move toward the stable fluid phase, analogous to results for attractive potentials with a single critical point^{29,30}. For example, for attractive well width $(c - b)/a = 0.2$, both C_1 and C_2 are metastable with respect to the crystal, whereas for $(c - b)/a > 0.7$ we find C_1 in the stable fluid phase.

The phase diagram also depends sensitively on the relative width of the shoulder b/a and on its relative height U_R/U_A . By decreasing b/a or by increasing U_R/U_A , T_2 decreases and becomes smaller than T_1 . This means that, in these cases, the high-density C_2 occurs below the temperature of the gas–liquid critical point, that is, C_2 is in the liquid phase and represents a LDL–HDL critical point, as in supercooled water^{6,7,11}.

The soft-core potential with the parameter sets we use displays no ‘density anomaly’ $(\partial V/\partial T)_P < 0$. This result is surprising at first because soft-core potentials have often been used to explain the density anomaly (see, for example refs 2 and 24). To understand this result, we consider the entropy S (the degree of disorder in the system) and the thermodynamic relation $-(\partial V/\partial T)_P = (\partial S/\partial P)_T = (\partial S/\partial V)_T(\partial V/\partial P)_T$. As, of necessity, $(\partial V/\partial P)_T < 0$, $(\partial V/\partial T)_P < 0$ implies $(\partial S/\partial V)_T < 0$, that is, the density anomaly implies that the disorder in the system increases for decreasing volume. For

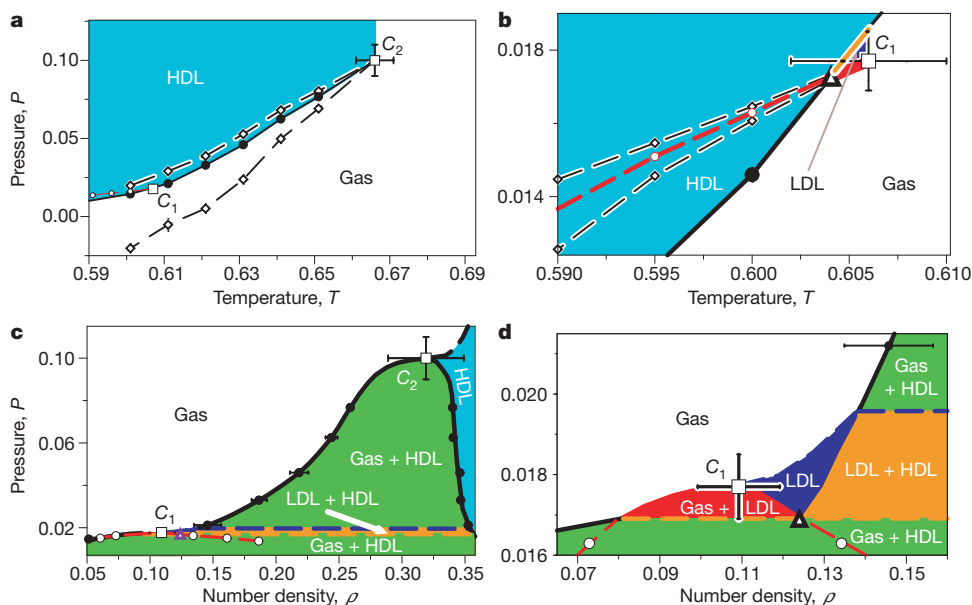


Figure 2 The phase diagrams, with coexistence lines and critical points resulting from MD simulations. **a**, **b**, P – T phase diagram. **b** is an enlargement of **a** in the vicinity of C_1 . Circles represent points on the coexistence lines: open circles are for the gas–LDL coexistence, filled circles for the gas–HDL coexistence. Lines are guides for the eyes. The solid black line is the gas–HDL coexistence line. The red line is the gas–LDL coexistence line. The solid red line is stable, and the dashed red line is metastable, with respect to the HDL phase. The orange line is the LDL–HDL coexistence line. The triangle represents the triple point. The projection of the spinodal line is represented in **a** and **b** by diamonds with dashed lines. The spinodal line is folded in this projection, with two cusps corresponding

to the two maxima in Fig. 1. Critical points occur where the coexistence lines meet these cusps. The critical point C_1 is for the gas–LDL transition, and C_2 is for the gas–HDL transition. **c**, **d**, P – ρ projections of **a** and **b**. The colours, symbols and patterns of coexistence lines, triple and critical points are the same as in **a** and **b**. Dashed blue, black and orange lines schematically represent isotherms at the temperatures of C_1 , C_2 and of the triple point, respectively. Both gas–LDL and gas–HDL coexistence lines show a local maximum, representing the estimates of C_1 and C_2 , respectively. In all the panels, where not shown, the errors are smaller than the symbol size. Units of P , T and ρ are as in Fig. 1.

example, this is the case for water. This is consistent with the negative slope dP/dT of the crystal–liquid transition line for water, that implies that $\Delta S/\Delta V < 0$ since, according to the Clausius–Clapeyron equation, $dP/dT = \Delta S/\Delta V$, where ΔS and ΔV are the entropy and volume differences between the two coexisting phases.

For our system, we expect the reverse: $(\partial V/\partial T)_P > 0$ so $(\partial S/\partial V)_T > 0$, consistent with the positive slope of the LDL–HDL transition line dP/dT (see Fig. 2b). We confirm that $(\partial S/\partial V)_T > 0$ by explicitly calculating S for our system by means of thermodynamic integration.

Our results show that the presence of two critical points and the occurrence of the density anomaly are not necessarily related, suggesting that we might seek experimental evidence of a liquid–liquid phase transition in systems with no density anomaly. In particular, a second critical point may also exist in liquid metals that can be described by soft-core potentials. Thus the class of experimental systems displaying a second critical point may be broader than previously hypothesized. □

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Strong radiative heating due to the mixing state of black carbon in atmospheric aerosols

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Aerosols affect the Earth's temperature and climate by altering the radiative properties of the atmosphere. A large positive component of this radiative forcing from aerosols is due to black carbon—soot—that is released from the burning of fossil fuel and biomass, and, to a lesser extent, natural fires, but the exact forcing is affected by how black carbon is mixed with other aerosol constituents. From studies of aerosol radiative forcing, it is known that black carbon can exist in one of several possible mixing states; distinct from other aerosol particles (externally mixed^{1–7}) or incorporated within them (internally mixed^{1,3,7}), or a black-carbon core could be surrounded by a well mixed shell⁷. But so far it has been assumed that aerosols exist predominantly as an external mixture. Here I simulate the evolution of the chemical composition of aerosols, finding that the mixing state and direct forcing of the black-carbon component approach those of an internal mixture, largely due to coagulation and growth of aerosol particles. This finding implies a higher positive forcing from black carbon than previously thought, suggesting that the warming effect from black carbon may nearly balance the net cooling effect of other anthropogenic aerosol constituents. The magnitude of the direct radiative forcing from black carbon itself exceeds that due to CH_4 , suggesting that black carbon may be the second most important component of global warming after CO_2 in terms of direct forcing.

This work was motivated by studies^{1–7} that found different black-carbon (BC) forcings when different BC mixing states were assumed. In one study⁷ the mixing state was found to affect the BC global direct forcing by a factor of 2.9 (0.27 W m^{-2} for an external mixture, $+0.54\text{ W m}^{-2}$ for BC as a coated core, and $+0.78\text{ W m}^{-2}$ for BC as well mixed internally). Because BC is a solid and cannot physically be well mixed in a particle, the third case was discarded as unrealistic, and it was suggested that the real forcing by BC probably fell between that from an external mixture and that from a coated core. Here I report simulations that were performed among multiple aerosol size distributions to estimate which of these two treatments, if either, better approximates BC forcing in the real atmosphere.

The global model that I used was GATOR-GCMM, which treated gas, aerosol, radiative, meteorological and transport processes (see Supplementary Information for details). Aerosol processes included emissions, homogeneous nucleation, condensation, dissolution, coagulation, chemical equilibrium, transport, sedimentation, dry deposition, and rainout among 18 aerosol size distributions, 17 size bins per distribution, one number concentration, and an average of seven mole concentrations per bin per distribution. The 18 distributions (Supplementary Information) consisted of four 'primary' size distributions (sea spray (A), soil (B), black carbon (E1) and organic

matter (F)) into which emissions occurred, one 'primary' distribution (sulphate (D)) into which homogeneous nucleation occurred, two additional BC distributions (E2 and E3) into which primary BC grew, 10 'binary' distributions (AB, AD, AE, AF, BD, BE, BF, DE, DF and EF) that resulted from heterocoagulation among A, B, D, E1, E2, E3 and F distributions, and a completely mixed distribution (MX) that resulted from all higher heterocoagulation interactions. Distributions A, B, D, E1 and F were initialized, and the aerosol population was relaxed with continuous emissions that were allocated to distributions A, B, E1 and F, homogeneous nucleation that was allocated to distribution D, and coagulation, growth, chemistry, transport and deposition among all distributions until a near global-scale steady state was obtained.

Figure 1 shows the variation with time of the globally averaged mass per cent of BC-containing particles that obtained a non-BC coating of various mass percentages when (simulation a; Fig. 1a) coagulation alone and when (simulation b; Fig. 1b) coagulation, condensation, dissolution and equilibrium water uptake were the only forms of internal mixing. In the simulation shown in Fig. 1a, coagulation caused about 35% by mass of initial and emitted externally mixed BC to obtain a coating > 20% by mass within five days. In the simulation shown in Fig. 1b, growth processes and coagulation together caused 63% of BC by mass to obtain a coating

> 20% by mass within five days. The difference indicates that growth and coagulation caused BC internal mixing at similar rates.

Coagulation has been calculated to affect the number and volume concentrations of particles primarily less than 0.2- μm diameter in urban air⁸. In that study, only one size distribution was considered. When multiple distributions are treated, the net effect of coagulation on the total number and volume concentrations of particles, summed over all distributions, is the same as that over one distribution if the initial number and volume concentrations are the same in both cases. The difference is that, in the multiple-distribution case, coagulation moves components among distributions (heterocoagulation), having a notable effect on particle composition not evident when only one distribution is simulated. Figure 2 shows such an effect. It shows the time-varying globally averaged mass per cent of BC in different distributions from the Fig. 1b simulation. Initially, all BC was externally mixed in distribution E1. BC then self-coagulated and grew into BC distributions E2 and E3 and heterocoagulated with sulphate, organic matter, soil and/or sea spray to form binary and higher mixtures. The difference between 100% and E1+E2+E3 in Fig. 2 represents the mass fraction of BC (50%) that heterocoagulated. Since this fraction is large, heterocoagulation appears to be important in the global-scale internal mixing of BC.

The mixing results that I report here seem to be consistent with

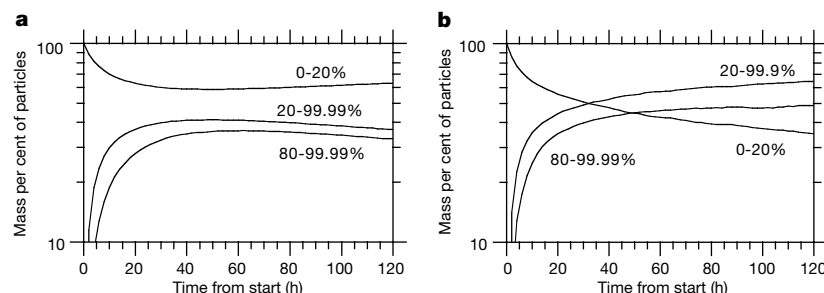


Figure 1 Coating of BC-containing particles over time. The figure shows the time-variation of the globally averaged mass per cent of BC-containing particles that obtain a non-BC coating of 0–20%, 20–99% and 80–99% by mass when (a) coagulation alone and (b) coagulation, condensation, dissolution, and equilibrium water uptake affect internal mixing. In both cases, emissions, homogeneous nucleation, transport, dry

deposition, sedimentation, and rainout of aerosols were also treated. Initially, 100% by mass of BC-containing particles were externally mixed (containing 0% coating by mass). The percentages in the figure were obtained by summing, over all BC-containing distributions, the mass of BC-containing particles of a given shell mass fraction and normalizing by the total mass of BC-containing particles.

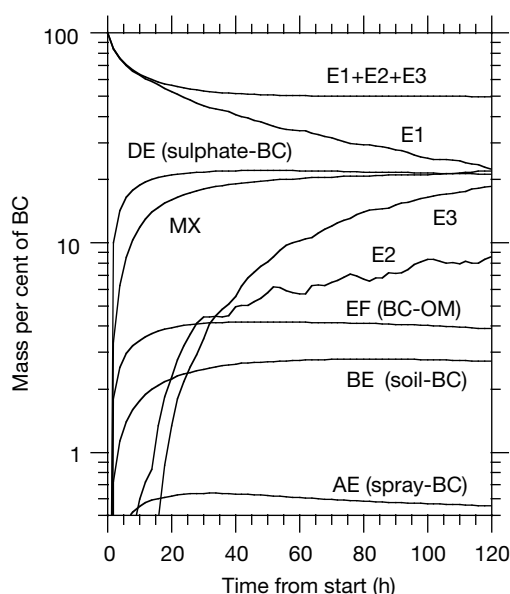


Figure 2 Shift of BC among particle distributions over time. The figure shows the globally averaged change in mixing state of BC among the 8 out of 18 size distributions in which BC was present, when coagulation, condensation, dissolution, and equilibrium water uptake affected internal mixing. Emissions, transport, dry deposition, sedimentation, and

rainout of aerosols were also accounted for. Initially, all BC was externally mixed in distribution E1. Coagulation moved BC from distribution E1 to binary mixtures of sulphate-BC, BC-organic matter (OM), soil-BC and spray-BC, to ternary and higher mixtures (MX) and to distributions E2 and E3. Growth also contributed to moving material from E1 to E2 and E3.

observations. Andreae *et al.*⁹ found that 80–90% of silicate particles over the Pacific Ocean between Ecuador and Hawaii contained sea spray. Here, by five days, 70–85% of soil particles in this region contained sea spray. Murphy *et al.*¹⁰ found that almost all particles $> 0.13 \mu\text{m}$ over the remote Southern Pacific Ocean contained sea spray. Here, $> 95\%$ of all near-surface particles in that region contained sea spray. Posfai *et al.*¹¹ found that almost all soot particles over the North Atlantic contained sulphate. Here, $> 93\%$ of BC-containing particles in this region contained sulphate.

Simulations were run to estimate the yearly and globally averaged radiative effects of treating BC in three ways: as a coated core in multiple distributions; as a single externally mixed distribution; and as a coated core in a single internally mixed distribution (Fig. 3). In the multiple-distribution case, modelled mid-visible optical depths (0.1–0.4) and single-scattering albedos (0.86–0.97) over the eastern United States and the western Atlantic Ocean during TARFOX were in the range of measured values (0.06–0.7 and 0.85–0.98, respectively; refs 12, 13). Modelled optical depths (0.16–0.44) in the Arabian Sea, and optical depths (0.07–0.17) and single-scattering albedos (0.88–0.94) in the Indian Ocean during INDOEX were similarly close to observations (0.2–0.4 (ref. 14), ≤ 0.1 (ref. 14) and 0.88–0.90 (ref. 15), respectively). Modelled mid-visible single-scattering albedos over biomass-burning regions of Brazil (0.86–0.94) were in the range of measurements obtained during the same time of year (0.82–0.94 (ref. 16), 0.79–0.95 (ref. 17). Modelled single-scattering albedos over biomass-burning regions of Africa (0.85–0.92) were close to those over biomass-burning regions of Brazil. The modelled annually averaged mid-visible optical depth over the global oceans (0.13) compares with a measured value of 0.12 (ref. 18).

Figure 3 shows that, within five days, the BC forcing from the multiple-distribution coated-core case was within 24% of that from the single-distribution coated-core case. This implies that the single-distribution coated-core assumption appears to be a better approximation of BC direct forcing than is the external-mixture assumption. As all studies to date have used the external-mixture assumption for determining lower bounds or average values of global direct forcing by anthropogenic aerosols, such lower bounds or average values must be higher (closer to zero) than all previous studies have predicted.

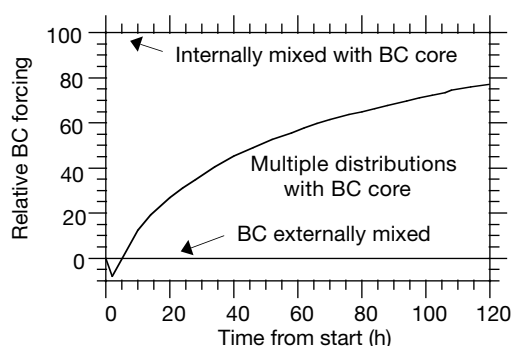


Figure 3 Time-dependent relative global BC direct forcing (at the tropopause) obtained when 18 size distributions were modelled. The relative forcing is $(F_{\text{mult}} - F_{\text{ext}}) / (F_{\text{int}} - F_{\text{ext}})$, where F_{mult} , F_{ext} and F_{int} are the forcings obtained with multiple distributions with BC as core, a single externally mixed BC distribution, and a single internally mixed distribution with BC as core, respectively. Each of the three forcing values at each time was calculated by averaging results from 24 simulations (6 days per year and 4 start times per day). For each simulation, the multiple-distribution forcing was obtained by initializing externally mixed distributions and relaxing towards a steady state with continuous emissions and nucleation of externally mixed particles and physical/chemical/deposition processes among all particles. The single externally and internally mixed distributions were derived from the multiple-distribution cases each time step in a mass-conserving manner to guarantee that the mass of BC and other components were exactly the same in each size bin each time step. Radiative calculations were then performed over the single distributions.

The final yearly averaged direct forcing due to BC in the external mixture, the multiple-distribution coated-core, and the single internally-mixed, coated-core distribution cases from Fig. 3 were 0.31, 0.55 and 0.62 W m^{-2} , respectively. The multiple-distribution BC direct forcing (0.55) falls between direct-forcing estimates for CH_4 (0.47 W m^{-2}) and CO_2 (1.56 W m^{-2}) from IPCC¹⁹. Thus, subject to uncertainties, BC may be the second most important component of global warming in terms of direct forcing, after CO_2 . For this reason, controls on BC emissions, not treated under the Kyoto Protocol of December 1997, need to be considered. Major sources of BC include the burning of diesel fuel, coal, jet fuel, natural gas, and biomass.

Previous studies have implied⁷ or suggested²⁰ that BC controls would be beneficial (studies have also shown that controls on non- CO_2 greenhouse-gas emissions would be beneficial²¹). The present study suggests that BC controls may be as—or more—beneficial than methane controls. Nevertheless, to obtain the true effect on rising global temperatures of controls on BC, CH_4 and CO_2 , comparative time-dependent simulations of the response of climate to these pollutants are needed. □

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Tropical climate changes at millennial and orbital timescales on the Bolivian Altiplano

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Tropical South America is one of the three main centres of the global, zonal overturning circulation of the equatorial atmosphere (generally termed the 'Walker' circulation'). Although this area plays a key role in global climate cycles, little is known about South American climate history. Here we describe sediment cores and down-hole logging results of deep drilling in the Salar de Uyuni, on the Bolivian Altiplano, located in the tropical Andes. We demonstrate that during the past 50,000 years the Altiplano underwent important changes in effective moisture at both

orbital (20,000-year) and millennial timescales. Long-duration wet periods, such as the Last Glacial Maximum—marked in the drill core by continuous deposition of lacustrine sediments—appear to have occurred in phase with summer insolation maxima produced by the Earth's precessional cycle. Short-duration, millennial events correlate well with North Atlantic cold events, including Heinrich events 1 and 2, as well as the Younger Dryas episode. At both millennial and orbital timescales, cold sea surface temperatures in the high-latitude North Atlantic were coeval with wet conditions in tropical South America, suggesting a common forcing.

The Salar de Uyuni (Fig. 1), located on the Bolivian Altiplano at an elevation of 3,653 m above sea level, is the world's largest salt flat. The salar was formerly occupied by a series of large lakes². The youngest was a shallow palaeolake, 'Coipasa', radiocarbon-dated³ between 11,500 and 13,400 calendar years before present (cal. yr BP). The youngest deep palaeolake, 'Tauca', was previously dated using carbonate fossils from outcropping sediments and carbonate bioherms (reefs) that mark past highstands. Published radiocarbon dates^{3,4} indicate that palaeolake Tauca existed from about 13,000 to 18,000 cal. yr BP (we judge the published U–Th dates of the same deposits to be less reliable than the published radiocarbon dates because of large corrections necessitated by the high values of excess thorium in the samples). Palaeolake Tauca attained a maximum depth of 140 m (ref. 5). The existence of an older deep palaeolake, 'Minchin', was postulated on the basis of two published radiocarbon dates (about 30,000 and 32,000 cal. yr BP; ref. 2) of shells from outcropping sediments. The evidence for the existence of these, and even older, palaeolakes on the Altiplano has been well summarized⁶.

In the summer of 1999 we drilled and continuously cored the

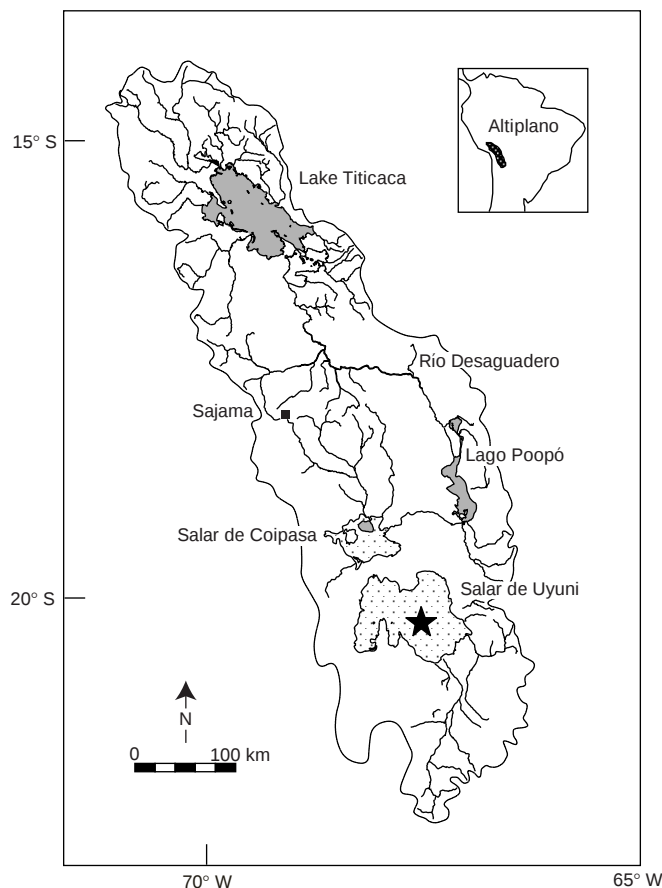


Figure 1 Map showing the location of the Altiplano and the Salar de Uyuni. During wet phases, Lake Titicaca overflows, and drains via the Rio Desaguadero into Lago Poopó. In

extremely wet periods, Lago Poopó can discharge into Salar de Coipasa and Salar de Uyuni. Star, location of the drill core; filled square, location of Volcan Sajama.

Salar de Uyuni to a depth of 220.6 m below the surface. The drill hole was located in the central portion of the salar (20° 14.97' S, 67° 30.03' W). The cased hole was subsequently logged at 10-cm intervals, using a tool (manufactured by Delta Epsilon, Inc.) that measures natural γ -rays, to a depth of 188.7 m subsurface. Here we present and discuss the stratigraphy and logging results of the radiocarbon-dated portion of the core, the upper 35 m.

The most important result of our study is the identification and dating (Table 1) of wet and dry events on the Altiplano (Fig. 2a) for the past 50,000 cal. yr. The wet events are signalled in the drill core by the muds containing abundant diatoms that indicate deposition in perennial lakes varying from shallow to deep and low to high salinity. The dry events are marked by salt deposits that consist mainly of halite (NaCl) and gypsum (CaSO₄). Salt textures indicate deposition in a variety of palaeoenvironments, ranging from perennial hypersaline lakes to annually desiccated saline pans similar to the modern Salar de Uyuni. Because the lacustrine muds have much higher values of natural γ -radiation than do the salt deposits, the continuous downhole record of natural γ -radiation (Fig. 2a) is a sensitive measure of changing effective moisture through time.

The shallowest peak of γ -radiation, indicating the former existence of a lake, is between 6.3 and 7.0 m subsurface. Incomplete recovery of sediment precluded direct dating of this interval, the probable equivalent of palaeolake 'Coipasa'. Our (extrapolated) age for this palaeolake is about 12,500 cal. yr BP, contemporaneous with the Younger Dryas cold event of high northern latitudes⁷. A γ -radiation minimum between 7.0 and 8.0 m records a dry period with salt deposition from about 12,500 to 14,900 cal. yr BP, contemporaneous with the Bølling–Allerød interstadial. Lacustrine muds were recovered between 8.0 and 14.0 m subsurface. We attribute these sediments to palaeolake Tauca, but our dates for this period extend to much older intervals than those previously published, ranging from 14,900 to 26,100 cal. yr BP. The two γ -radiation maxima within this interval, dated at 14,900–16,600 and 24,300–26,100 cal. yr BP, coincide respectively with Heinrich events 1 and 2a⁷. Underlying the Tauca sediments, between 14.0 and 20.4 m subsurface are two minor lacustrine muds marked by smaller γ -radiation maxima, dated at 28,200–30,800 and 31,800–33,400 cal. yr BP, that coincide with two sea surface temperature (SST) maxima observed in the subtropical North Atlantic⁷. These muds are interbedded with halite that formed during dry periods. The second youngest major lacustrine interval extends from 20.4 m to 33.3 m subsurface. A sample from near the top of this interval yielded a reliable age of about 42,000 cal. yr BP, yielding an interpolated age for the top of the interval of 38,100 cal. yr BP. Deeper samples were beyond the range of radiocarbon dating. We attribute this interval to palaeolake Minchin; thus, palaeolake Minchin is much older than previously believed. Underlying the Minchin bed is a 7-m-thick salt bed that is, in turn, underlain by a third major, as yet undated, lacustrine interval.

A recent global climate modelling experiment⁸, utilizing new sea surface palaeotemperature estimates⁹, yielded a mean annual temperature lowering of 5 °C relative to modern for the Last Glacial Maximum (LGM) in this part of the tropical Andes. Studies of the depression of snowline elevation have concluded variously that the mean annual temperature change for the LGM in this part of the Andes ranged anywhere from 2–3 °C (refs 10, 11) to 5–9 °C (ref. 12). Model calculations of the amount of precipitation needed to maintain large palaeolakes on the Altiplano against desiccation by evaporation¹³ necessitate about 30% higher-than-modern precipitation, even with temperatures 5 °C lower than modern. We conclude that the lacustrine episodes observed in our drill core were not produced solely by lower temperatures; their formation also required significantly higher amounts of precipitation on the Altiplano than today.

The austral summer is the season of maximum precipitation in the southern tropics of South America (including the Altiplano)—

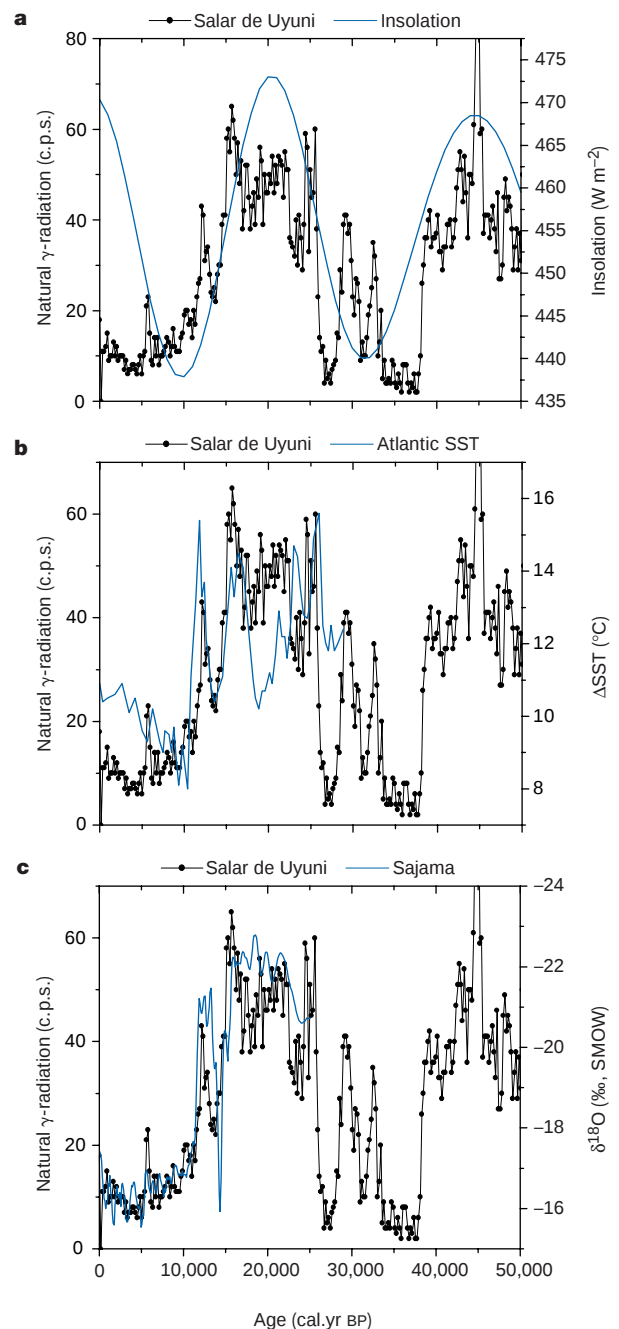


Figure 2 Logging data from the Salar de Uyuni compared to other reconstructed palaeoclimatic time series. The figure compares natural γ -radiation in the salar drill hole, our proxy for effective moisture, with calculated summer insolation, reconstructed records of sea surface temperature (SST) gradients in the equatorial Atlantic sector, and the record of stable oxygen isotopes in the Sajama ice cap. **a**, Natural γ -radiation (black line and dots) versus calendar age determined by radiocarbon measurement (Table 1). Natural γ -radiation is mostly produced by the decay of radioactive K, U and Th, elements that are more concentrated in the lacustrine muds than in the salt deposits. The high values of natural γ -radiation centred at 45,000 cal. yr BP are due to volcanic ash. Blue line, palaeoinsolation at 15° S latitude calculated for the month of January¹⁵. **b**, Natural γ -radiation (black line) versus calendar age. Blue line, Δ SST, the difference in reconstructed SST between the western tropical North Atlantic¹⁸ and the eastern subtropical North Atlantic⁷. Values of reconstructed SST (using the calibration of Muller *et al.*²³) and age were interpolated from both studies, then differenced. **c**, Natural γ -radiation (black line) versus calendar age. Blue line, the smoothed record (3-point moving average of the 100-year average record; L. Thompson, personal communication) of $\delta^{18}\text{O}$ of ice in the Sajama ice cap²².

on the Altiplano an average of 75% of the total annual precipitation falls in the months of December–March. During the austral summer, atmospheric water vapour, ultimately derived from the tropical Atlantic Ocean, is advected across the Amazon basin to the centre of deep convection developed in the Gran Chaco region to the east of the Altiplano. This climatological system has been referred to as the South American summer monsoon (SASM)¹⁴. We propose that in the past the first-order control on the variability of precipitation on the Altiplano was variability of the SASM produced by changes in summertime insolation. Indeed, the main wet and dry phases on the Altiplano occurred, respectively, in phase with summertime (January) insolation maxima and minima (Fig. 2a), calculated for 15°S latitude from Earth's known orbital variations¹⁵. For example, the intervals 14,900–26,100 cal. yr BP (including the LGM) and 38,100 to ~50,000 cal. yr BP were periods of maximum wetness and maximum insolation on the Altiplano. An apparent exception to this hypothesized control is the lack of a modern lake in the Salar de Uyuni in the present time of high summer insolation. However, the late Holocene is a relatively wet period on the Altiplano—Lake Titicaca has been at or near its overflow level since about 3,500 cal. yr BP (ref. 16) and small lakes (such as modern Lago Poopó) have existed on the central Altiplano during much of this period¹⁷. Also, the late Holocene is warmer than the LGM, contributing to a higher rate of evaporation. It is clear that the SASM did not decrease in intensity during the LGM, despite colder tropical SST. Instead, increased land–sea temperature gradients and increased interhemispheric meridional SST gradients⁹, combined with peak wet-season insolation, resulted in an enhanced SASM and enhanced precipitation in much of tropical South America.

Superimposed upon the first-order orbital control of moisture, we observed many high-amplitude, but shorter (millennial) duration peaks of γ -radiation indicative of the former presence of lakes on the Altiplano, even during time periods calculated to have relatively low summertime insolation. Allowing for reasonable dating errors, each peak corresponds with an SST minimum

reconstructed for the subtropical eastern North Atlantic (38°N, 10°W) SST⁷. When eastern North Atlantic SST is subtracted from western North Atlantic (12°N, 61°W) SST¹⁸, the resulting Δ SST (Fig. 2b) is a useful measure of palaeoatmospheric dynamics (we note that there are also very similar, but not as well dated, SST reconstructions¹⁹ in the eastern tropical Atlantic at 21°N, 19°W and 19°N, 20°W). In the modern ocean, high values of this index would be expected to correlate with enhanced northeast trade winds²⁰ and, as observed, increased advection of moisture to the Amazon and the Altiplano²¹. The Salar de Uyuni record provides the best demonstration to date that millennial-scale Heinrich events occurred in southern tropical South America, where they are manifested as wet, not cold, periods.

The oxygen isotope record of the nearby (Fig. 1) ice cap of Volcan Sajama²² has a similar structure to the Salar γ -radiation record (Fig. 2c), bolstering the argument that $\delta^{18}\text{O}_{\text{ice}}$ is inversely correlated with precipitation amount (or runoff fraction)¹⁰. The base of the ice core coincides in age with the basal sediments of palaeolake Tauca (26,100 cal. yr BP), suggesting that the preceding dry phase had resulted in the complete loss of ice on Sajama (and perhaps elsewhere in the western Andean Cordillera).

We have reconstructed the timing and direction of large changes of precipitation on the Altiplano over the past 50,000 yr. The general agreement between the timing of the longest wet phases and periods of above-average summer insolation supports a first-order orbital control of the intensity of the SASM. Furthermore, all wet phases at both orbital and millennial scale coincide with anomalously large zonal SST gradients (warm west and cold east) across the equatorial Atlantic Ocean, the ultimate moisture source for the Altiplano. □

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Table 1 Radiocarbon dates of sediments from the Salar de Uyuni core

Sample	AMS no.	$\delta^{13}\text{C}$ (‰ PDB)	Radiocarbon age (yr BP)	Calibrated age (yr BP)
SU 6.91	OS-26880	-16.0	10,980 ± 55	13,074
SU 8.47	OS-21541	-13.9	13,800 ± 100	16,560
SU 9.78	OS-26878	-13.9	13,840 ± 65	16,606
SU 10.20	OS-21542	-13.6	18,920 ± 250	22,450
SU 13.35	OS-21543	-13.4	19,250 ± 190	22,830
SU 15.14	OS-21544	-13.5	23,600 ± 190	27,750
SU 15.72	OS-21545	-13.4	24,100 ± 200	28,320
SU 16.22	OS-21546	-13.3	24,000 ± 800	28,210
SU 17.09	OS-21547	-13.4	23,900 ± 190	28,100
SU 17.70	OS-21548	-15.9	32,600 ± 410	37,830
SU 18.37	OS-21549	-15.5	31,500 ± 480	36,630
SU 22.78	OS-21550	-17.6	36,300 ± 590	41,840
SU 24.04	OS-21751	-15.4	>45,000	>51,000
SU 27.25	OS-21752	-17.9	>45,000	>51,000
SU 28.15	OS-21753	-17.7	>45,000	>51,000
SU 28.40	OS-21754	-15.9	>45,000	>51,000
SU 30.74	OS-21755	-15.9	>45,000	>51,000

Radiocarbon dates were determined by accelerator mass spectrometry (AMS) on the total organic carbon fraction of the lacustrine muds or salts. These dates were calibrated using Calib 4.1.2^{24,25} for samples less than 24,000 cal. yr BP, and using a quadratic equation²⁶ for older samples. Our age–depth model is a linear, least-squares fit assuming a surface age of zero. Ages are corrected for stable isotopic fractionation, but not for reservoir effects. Large reservoir effects are present in some groundwater-dominated lakes of the Chilean Altiplano²⁷ and have been suggested for palaeolake ‘Coipasa’⁴. We doubt, however, that significant reservoir effects were present in the larger palaeolakes. For example, the large, deep basin of modern Lake Titicaca has no appreciable reservoir effect²⁸, although a 250-year reservoir correction is needed for the shallow, semi-isolated basin of the modern lake⁶. The major lake phases of the Salar de Uyuni coincided with higher-than-modern levels of Lake Titicaca, hence greater-than-modern throughflow rates of water in that lake²⁹. At those times, Lake Titicaca was deep, fresh and overflowing to the south, contributing about one-third of the total input to palaeolakes ‘Minchin’ and ‘Tauca’²⁹. The large surface area-to-volume ratio of these two palaeolakes would have promoted rapid exchange of dissolved CO₂ with atmospheric CO₂. Finally, there is little carbonate rock in the watershed of the Salar de Uyuni. Under these conditions it is difficult to envision a significant flux of non-atmospheric carbon into the palaeolakes, and we conclude that any reservoir effect is small.

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Upper-mantle dynamics revealed by helium isotope variations along the southeast Indian ridge

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Helium isotope variations in igneous rocks are important for relating isotopic heterogeneity to convective mixing in the Earth's mantle. High $^3\text{He}/^4\text{He}$ ratios at many ocean islands, along with lower and relatively uniform values in mid-ocean-ridge basalts (MORBs), are thought to result from a well mixed upper-mantle source for MORB and a distinct deeper-mantle source for ocean island basalts¹. At finer scales, $^3\text{He}/^4\text{He}$ variations along mid-ocean ridges have been related to underlying mantle heterogeneity^{2,3}, but relationships between the scales of geochemical segmentation and mantle convection remain enigmatic. Here we present helium isotope data for MORB glasses recovered along ~5,800 km of the southeast Indian ridge, and develop an approach to quantitatively relate spatial variations in geochemical and geophysical parameters at the Earth's surface. A point-to-point correlation analysis reveals structure in the helium isotope data at length scales of ~150 and ~400 km that appears to be related to secondary convection in the underlying mantle.

The southeast Indian ridge (SEIR; Fig. 1) stretches from the Rodrigues triple junction (25.6°S, 70.1°E) to the Macquarie triple junction (62°S, 151°E). Between 76° and 78°E it crosses the Amsterdam–St Paul plateau (ASP), a pronounced swell associated with relatively hot mantle upwelling beneath Amsterdam and St Paul islands³, while between 120° and 128°E it crosses the Australian–Antarctic discordance (AAD), a region of deep bathymetry (~4,000 m) associated with relatively cold mantle and low

melt production⁴. From 88° to 120°E, the increase in depth of the ridge axis, and the morphological transition from an axial high to an axial valley, imply that melt production rate and crustal thickness decrease eastwards along axis, despite the relatively constant full spreading rate of 70–75 mm yr^{−1} (ref. 5). On the basis of petrologic analyses of more than 3,000 basalt glasses^{6–9}, there is an inferred gradient in mantle temperature of ~80–150 °C from east to west. The hottest mantle is present beneath the ASP, and the coldest mantle beneath the AAD region. Given the constant spreading rate and the absence of large transform offsets and nearby hotspots, this portion of the SEIR provides a unique opportunity to study the geochemical consequences of along-axis variations in upper-mantle temperature.

We analysed helium isotopes following *in vacuo* crushing of basalt glass¹⁰, and report the helium isotope composition relative to the atmospheric $^3\text{He}/^4\text{He}$ ratio (R_A). (Data are available as Supplementary Information.) Variations in $^3\text{He}/^4\text{He}$, axial depth and Fe_8 are shown against distance along the SEIR in Fig. 2. (Fe_8 is the FeO content in wt%, corrected for crystal fractionation to 8% MgO , and may be used as an indicator of the mean depth of mantle melting¹¹). Between distances of ~1,000 and 3,000 km east of the ASP (that is, from the axial depth minimum at 88°E to the AAD; Fig. 2), there is a long-wavelength eastward decrease in $^3\text{He}/^4\text{He}$ and Fe_8 , and an increase in axial depth which may trace sub-ridge mantle flow down a thermal gradient⁵. Near the ASP, $^3\text{He}/^4\text{He}$ ratios extend above 14 R_A due to the presence of the Amsterdam–St Paul hotspot³. In the AAD, $^3\text{He}/^4\text{He}$ ratios are as low as 6.2 R_A , and are among the lowest values yet observed in MORB away from the influence of subduction zones¹².

There is a good $^3\text{He}/^4\text{He}$ – Fe_8 covariation along much of the SEIR. Because the peaks in $^3\text{He}/^4\text{He}$ and Fe_8 do not occur in precisely the same locations, we computed Spearman's rank correlation coefficient (r_s) as a non-parametric test of the significance of the correlation. We excluded samples on top of the ASP to remove hotspot effects. For the remaining data ($n = 91$) $r_s = 0.65$, well above the critical value of 0.21 for a 0.05 significance level (in a two-tailed test with 90 degrees of freedom), and so the null hypothesis that the $^3\text{He}/^4\text{He}$ – Fe_8 relationship is random can be rejected. The covariation of $^3\text{He}/^4\text{He}$ and Fe_8 therefore suggests that higher $^3\text{He}/^4\text{He}$ is associated with a higher mean pressure of melting¹¹. One possible explanation for the $^3\text{He}/^4\text{He}$ – Fe_8 covariation is a layered upper mantle having higher $^3\text{He}/^4\text{He}$ ratios at depth. Mantle which is

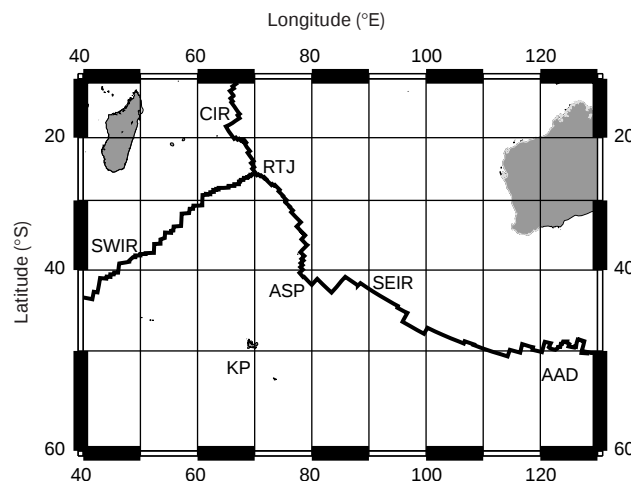


Figure 1 Location map of Indian Ocean features. SEIR, southeast Indian ridge; SWIR, southwest Indian ridge; CIR, central Indian ridge; RTJ, Rodrigues triple junction; ASP, Amsterdam–St Paul plateau; AAD, Australian–Antarctic discordance; KP, Kerguelen plateau.

hotter and begins to melt deeper will produce aggregated MORB melts having higher values of Fe_8 (ref. 11). According to this first explanation, $^3\text{He}/^4\text{He}$ values will also be higher due to a proportionally greater sampling of the deep upper mantle. Another possible origin for the $^3\text{He}/^4\text{He}$ – Fe_8 covariation is from melting of discrete heterogeneities, having lower solidus temperatures, that are embedded within upper-mantle peridotite. Such heterogeneities could, for example, occur as blobs or veins of garnet pyroxenite having low $^3\text{He}/^4\text{He}$, due to their origin from recycled crust¹³. Garnet pyroxenite melts preferentially to surrounding mantle peridotite. However, its contribution to erupted basalt is significantly less when magma genesis begins at a higher mantle temperature and pressure, because the pyroxenitic melts become effectively diluted by partial melts of peridotite¹⁴. According to this second explanation, hotter mantle would begin to melt deeper and would also lead to aggregated MORB melts that have higher values of Fe_8 and $^3\text{He}/^4\text{He}$. This model implies that some highly depleted

(pyroxenite-free) regions of the upper mantle may have $^3\text{He}/^4\text{He}$ ratios above $9R_A$. One of the few examples of such high $^3\text{He}/^4\text{He}$ ratios in ultra-depleted MORB glass comes from the Garrett transform on the southern East Pacific Rise¹⁵, where a MORB lava with $\epsilon_{\text{Nd}} = +11$ has $^3\text{He}/^4\text{He} = 9.7R_A$.

Superimposed on the long-wavelength gradients along the SEIR, there are several peaks in $^3\text{He}/^4\text{He}$ and Fe_8 that range between 200 and 500 km in basal width (Fig. 2). These peaks are most prominent near the ASP, and at 88° E, 97° E and 111° E (at along-axis distances of 910, 1,800 and ~2,850 km, respectively; Fig. 2). The latter three peaks occur near the centres of the zero-order, regional segmentation of the ridge (Fig. 2) defined by the persistence of fracture zone anomalies in satellite gravity data. This segmentation reveals several coherent tectonic units, up to ~900 km in along-axis width, that have been stable since reorientation of the SEIR when it migrated over the Kerguelen plume 38 Myr ago (ref. 16).

To evaluate quantitatively the length scales of helium isotope variation along the SEIR, we applied a spatial correlogram analysis, similar to approaches used previously to study magma mixing¹⁷. The correlation coefficient R is calculated between every pair of points at a separation distance r . For each point pair, $R(r)$ is given by the product of the deviations in $^3\text{He}/^4\text{He}$ from the population mean, normalized to the population variance¹⁸. For n sample points, the total number of point pairs is $N = n(n-1)/2$; for the 120 sample localities along the SEIR there are 7,140 sample pairs. A value of $R(r)$ close to 1 indicates that a $^3\text{He}/^4\text{He}$ value above the population average at a given location is likely to be associated with an above-average value at a distance r away. (Similar arguments hold for below-average values.) $R(0)$ is equal to unity by definition. A value of $R(r)$ close to zero implies a random relationship between $^3\text{He}/^4\text{He}$ values at points located a distance r apart. A value of $R(r)$ close to –1 implies an anticorrelated relationship. For any mixture which is not perfectly homogeneous, $R(r)$ will be greater than 0 at small values of r because points close together will usually be from the same ‘clump’¹⁸. The lowest value of r at which $R(r)$ goes to zero is denoted as r^* .

The correlogram ($R(r)$ versus r) can be used to define a scale of segregation¹⁸ similar to conceptualizations for the scale of turbulence in fluid flow¹⁹. The scale of segregation, L , is the integral of $R(r)$ from $r = 0$ to $r = r^*$, and is related to the size of clumps within a mixture. Within the Earth's interior, such clumps must vary in size and shape and their boundaries may be diffuse²⁰. We have no *a priori* information on their dimensions, so it is not possible to refer to an ‘average size’ of clumps. We introduce the L concept because it is a quantitative estimate of size that can be precisely defined from spatially referenced geochemical data. (We note that there are three dominant ‘scales of mantle convection’ inferred from seismic tomography, topography and gravity studies²¹. These dominant length scales occur at spherical harmonics degree 2 (~16,000 km) and degree 6 (~6,000 km), and at a smaller scale between 400 and 1,000 km. The length scales of geochemical variation in the mantle should be related to these convective scales, although not necessarily in a one-to-one manner.)

The correlogram method is strictly only applicable to stationary data; that is, data which show no trend in mean level over the length of observation. Because there is a long-wavelength gradient in the $^3\text{He}/^4\text{He}$ baseline along the central portion of the SEIR, we applied the spatial correlogram approach both to the measured $^3\text{He}/^4\text{He}$ ratios, and to computed regression residuals from a linear fit of $^3\text{He}/^4\text{He}$ versus along-axis distance. Both the correlogram for the $^3\text{He}/^4\text{He}$ results and that for the residuals show the expected decrease in $R(r)$ with separation distance (Fig. 3). The values of r^* and L for the raw data are 1,600 km and 620 km, respectively, but these estimates are strongly affected by the existence of the regional $^3\text{He}/^4\text{He}$ gradient, as confirmed by simple forward models. For the ‘detrended’ data (that is, the regression residuals) r^* and L are ~400 km and ~150 km, respectively. These distances accurately

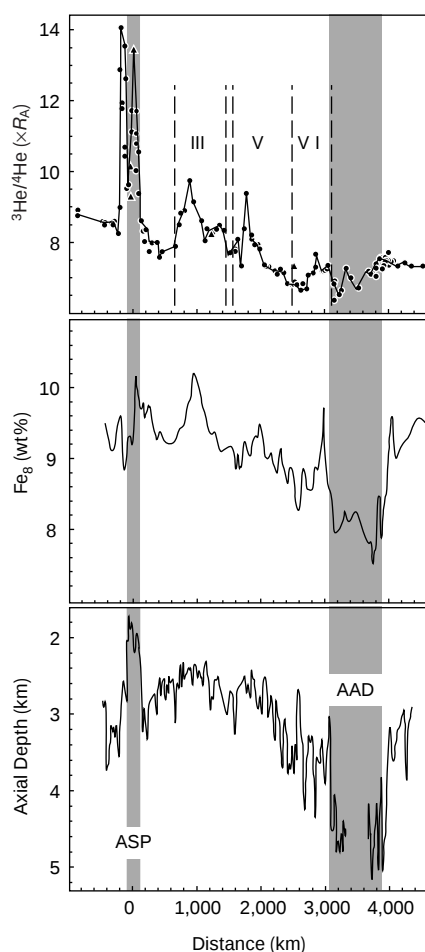


Figure 2 $^3\text{He}/^4\text{He}$, Fe_8 and axial depth versus distance along the SEIR. Basalt glasses have been recovered from the SEIR by a number of seagoing expeditions. From segments immediately northwest of the Amsterdam–St Paul plateau (ASP) to the Australian–Antarctic discordance (AAD), all sampling has been precisely located in the neovolcanic zone based on multi-beam bathymetry and magnetic surveys. Average sample density for $^3\text{He}/^4\text{He}$ across this region of ~4,000 km is 1 sample every 45 km. Distance is calculated from the pole of plate rotation (13.2° N, 38.2° E)²⁹ and is shown relative to St Paul Island (38.70° S, 77.55° E). The ASP and AAD regions are depicted as shaded bands. The dashed lines and Roman numerals delineate the zero-order tectonic segmentation from satellite gravity analysis¹⁶. The line shown for Fe_8 is the five-point running mean. Triangles represent lavas from seamounts and off-axis lava fields. Helium isotope data are from this study and from refs 3 and 30.

reflect the length scales of $^3\text{He}/^4\text{He}$ variation that are qualitatively discernible within the regional trend along the SEIR (Fig. 2).

Because these values of r^* and L are significantly larger than the average sampling density of ~ 45 km, they do not represent random variations. The value of $r^* = 400$ km resembles the largest scale (zero order) tectonic segmentation of the ridge, delineated by fracture zones that have persisted throughout the spreading history of the SEIR¹⁶. Length scales of the order of 10^2 km are similar to those of hypothetical, secondary convective ‘cells’ in the underlying mantle. For example, undulations in the geoid for the Pacific plate, ranging in wavelength from 200 km near the ridge to 400–600 km far away, have previously been ascribed to convective rolls in the uppermost mantle^{22,23}. Such cellular structures would be aligned roughly orthogonal to the ridge axis. If such secondary convection is present beneath the SEIR, its geometry may be a remnant from the passage of the ridge over the Kerguelen plume about 38 Myr ago (ref. 24), similar to features that form in the wake of plumes in numerical models of mantle convection²⁵. Alternatively, secondary convection might result from a thermal feedback induced by fracture zones, where patterns of upper-mantle convection are established during the initiation of spreading or continental breakup. A further possibility is that it might simply be an intrinsic feature of the inferred eastward mantle flow toward the AAD in this part of the Indian Ocean, the so-called ‘Richter rolls’²⁶.

Mantle convection is undoubtedly unsteady on a geological timescale. The simplest form of this unsteadiness is one in which convective cells oscillate or pulsate slightly²⁷. Significantly, this tends to produce some lateral mass transport between cells. Based on our correlogram analysis, the characteristic length scale for tracer (helium) transport is of the order of 150–400 km in this region of the Indian Ocean mantle. ‘Mixing’ within cells of this length would be significantly faster than ‘dispersion’ between cells, and the relative importance of these transport phenomena would mainly depend on the regional Rayleigh number.

Our results provide good evidence for some control of along-axis

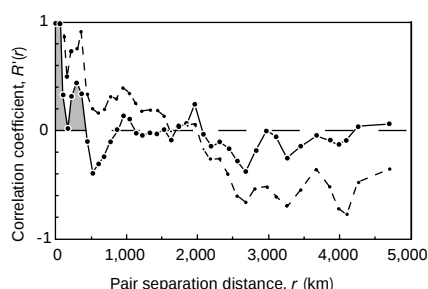


Figure 3 Spatial correlogram for $^3\text{He}/^4\text{He}$ variations along the SEIR. The correlation coefficient $R(r)$ is

$$R(r) = \frac{(\bar{c}_i - \bar{c})(\bar{c}_j - \bar{c})}{\sigma^2}$$

where c_i and c_j are the $^3\text{He}/^4\text{He}$ ratios at locations i and j , respectively, $\bar{c}$ is the population mean, σ^2 is the population variance, r is the distance between points i and j , and the overbar designates an ensemble average over all pairs. The mean value of $R(r)$ has been plotted for a constant number of point pairs per distance interval. This constant population method produces similar results to one using bins of constant width, but provides a more robust correlogram at large r . The correlograms shown are for the full data set, with 120 sample locations (7,140 point pairs) and $r_{\text{max}} = 5,843$ km, using 40 distance bins each with 178 members. The mean $^3\text{He}/^4\text{He}$ is $8.30 R_A$, and the variance is $2.43 R_A$. The dashed line shows the correlogram for the raw $^3\text{He}/^4\text{He}$ data, and the solid line shows the correlogram for regression residuals in which the regional $^3\text{He}/^4\text{He}$ gradient has been removed by a linear fit. For this detrended $^3\text{He}/^4\text{He}$ case, the segregation length scale (L) shown by the shaded area is ~ 150 km, and r^* (see text) is ~ 400 km.

geochemical variations by secondary convection patterns in the upper mantle. Spatial correlogram analysis provides a way to investigate geochemical variations at local, regional and global scales; this technique also has the potential to link quantitatively such variations to geophysical patterns—such as those detected with seismic tomography²⁸. □

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Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution

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The origin of the ratites, large flightless birds from the Southern Hemisphere, along with their flighted sister taxa, the South American tinamous, is central to understanding the role of plate tectonics in the distributions of modern birds and mammals. Defining the dates of ratite divergences is also critical for determining the age of modern avian orders^{1–6}. To resolve the ratite phylogeny and provide biogeographical data to examine these issues, we have here determined the first complete mitochondrial genome sequences of any extinct taxa—two New Zealand moa genera—along with a 1,000-base-pair sequence from an extinct Madagascan elephant-bird. For comparative data, we also generated 12 kilobases of contiguous sequence from the kiwi, cassowary, emu and two tinamou genera. This large dataset allows statistically precise estimates of molecular divergence dates and these support a Late Cretaceous vicariant speciation of ratite taxa, followed by the subsequent dispersal of the kiwi to New Zealand. This first molecular view of the break-up of Gondwana provides a new temporal framework for speciation events within other Gondwanan biota and can be used to evaluate competing biogeographical hypotheses.

Ratites and tinamous (together described as palaeognathes) share a primitive skull structure (the palaeognathous palate) and are taxonomically separated from all other living birds (neognathes). Ratites include the rhea (*Rhea americana*, *Rhea pennata*, South America), ostrich (*Struthio camelus*, Africa and formerly Eurasia), emu (*Dromaius novaehollandiae*, Australia), cassowary (*Casuarius casuarius*, *Casuarius bennetti*, *Casuarius unappendiculatus*, Australasia) and kiwi (*Apteryx mantelli*, *Apteryx owenii*, *Apteryx haasti*, New Zealand), as well as the recently extinct moa (11 spp., New Zealand) and elephant-bird (~3 spp., Madagascar)^{1,2}. Ratite fossils are found on all the Southern continents formed by the Cretaceous break-up of the supercontinent Gondwana^{1,7}, and are commonly used as an example of vicariant speciation^{1,8}. This hypothesis has been challenged by the discovery of small flighted palaeognathe fossils (lithornids) from the early Tertiary of the Northern Hemisphere⁹, and suggestions that modern birds represent a post-Cretaceous radiation⁷.

Despite much research, the nature of the basal ratite divergences remains unclear, confounding biogeographical interpretations. An influential early morphological study suggested that the New Zealand ratites were monophyletic, and the first to diverge¹ (but see ref. 10). However, initial molecular studies suggested that the kiwi, emu and cassowary were a derived group, with either the rhea or ostrich as the basal divergence^{2,3,6}. Ancient DNA methods allowed short mitochondrial DNA (mtDNA) sequences from several moa taxa to be compared; these indicated that the New Zealand ratites were not monophyletic, and that the moa fell between a basal rhea branch and the ostrich^{3,11}. This result was challenged by further mtDNA data from living taxa^{6,8}, and remains unresolved because of

the small amounts of sequence data available, particularly for the extinct moa.

To resolve this issue we used ancient DNA techniques to generate contiguous sequences of the complete mitochondrial genomes of two moa genera, *Emeus crassus* and *Dinornis giganteus*, as a series of 400–600 base-pair (bp) amplifications from subfossil bones 1,300–1,500 years old (see Methods). Competitive polymerase chain reaction (PCR) assays indicated that mtDNA was preserved at around 0.3–1.5 million copies per gram of bone in the specimens, roughly three orders of magnitude higher than the Neanderthal (2,500–3,750 copies per g) and Ice Man DNA (8,600 copies per g), but similar to some Hohokam mummies from the US southwest (2 million copies per g)^{12,13}. The high concentration of moa mtDNA indicates that amplified sequences are unlikely to be influenced by damage to individual template molecules^{12,13}. This was confirmed by cloning experiments (Table 1) where sequencing discrepancies occurred at rates comparable to modern taxa (~2 errors per 1,000 bp) and consisted mainly of singletons. Sequencing experiments replicated in Barcelona and London were identical and cloning error rates were similar to those in Oxford. In the control regions of both moa, a 90-bp section could not be sequenced unambiguously because flanking thymidine homopolymers caused sequencing reactions to stutter. A putative nuclear copy of mitochondrial sequences (numt) was also detected in *Dinornis*. The putative numt featured two indels of 82 bp and 12 bp, but was otherwise identical to the mtDNA sequences.

We also used long-range PCR to amplify and sequence all the mitochondrial protein coding genes (apart from ND6) of the emu, cassowary, kiwi and two tinamou genera. The data were aligned

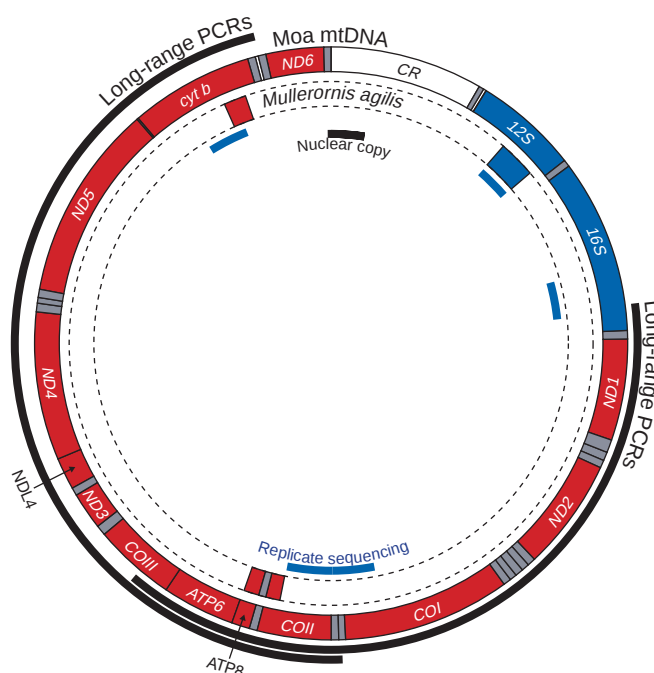


Figure 1 Mitochondrial genome arrangement for *Dinornis giganteus*. The moa mtDNA gene arrangement is similar to published rhea and ostrich sequences, and is 16,997 bp in length. Sequences of *Dinornis* and *Emeus* independently replicated in London and Barcelona, respectively, are indicated and the primers used were 12S (London and Barcelona), 1753FH/2150RH; 16S and COII (Barcelona), 3348Fb/3797RH, 8320Fb/8807Rb; COI and Cyt b (London), 7807FH/8325Rb, 15303Fb/15783Rb. Sequences of *Mullerornis* were obtained for 12S (1753FH/1985Rb; 1856Fb/2020Rb; and 1999Fb/2150RH), COII (8861Fb/9036RH), ATP8 (9043Fb/9241RH) and Cyt b (15671FH/15902RH). 1856Fb/2020Rb sequences were replicated in London. Long-range PCR and primers of extant taxa were used to minimize the risk of numts, and used primers 03725FH-16SFor.2/10218RH-COIII-LR, and 08171FH-tSer-LR/16120Rb-tPro-LR.

with the two moa sequences, and the published rhea, ostrich and chicken sequences (see Methods). After ambiguous sites and gaps were removed, the remaining 10,767 nucleotides were used for phylogenetic analysis. Identical maximum-likelihood trees (Fig. 2) were generated by exhaustive heuristic and constrained searches. These confirmed that the moa and kiwi are not monophyletic, and represent separate ratite invasions of New Zealand^{3,11,14}. Although bootstrap values are relatively low, nearest-neighbour interchange values show that the tree is locally stable and that the ostrich is never basal. Parametric bootstrapping rejected the two alternative ratite phylogenetic hypotheses at the $P < 0.001$ significance level (Fig. 2). Maximum-likelihood analyses of a short (1000-bp) dataset featuring the elephant-bird (see Methods and Supplementary Information) placed it among the derived ratite taxa (Fig. 2). Although these data do not allow precise placement or an estimate of divergence date, the elephant-bird is clearly not the result of a recent divergence from the ostrich, or any other ratite lineage.

Molecular clock tests revealed that the sequences have evolved in a

clock-like manner, with the exception of the ostrich, which has an elevated rate. When the ostrich branch was allowed a different rate, the likelihood ratio of 0.449 is consistent with rate constancy for the other taxa ($P = 0.92$). The large sequence dataset, and clock-like behaviour of the ratite lineages, provides an opportunity to integrate statistically precise divergence time estimates with the geological history of Gondwana. There are two potential calibration points: first, Mid to Late Oligocene fossils which show that the emu and cassowary lineages had clearly separated sometime before 25 Myr ago (ref. 15), probably around 30–35 Myr (W. Boles, personal communication); second, the geological split between New Zealand and Australia/Antarctica around 82–85 Myr (refs 16, 17), which has been related to the basal separation of the moa from the other ratite taxa^{14,18,19}. Unfortunately, like many terrestrial avian groups the rest of the ratite fossil record is relatively poor, with only the rhea and ostrich known from the early Tertiary^{4,9,20}. These fossils can provide only a minimum age for these long unbranched lineages (Fig. 2).

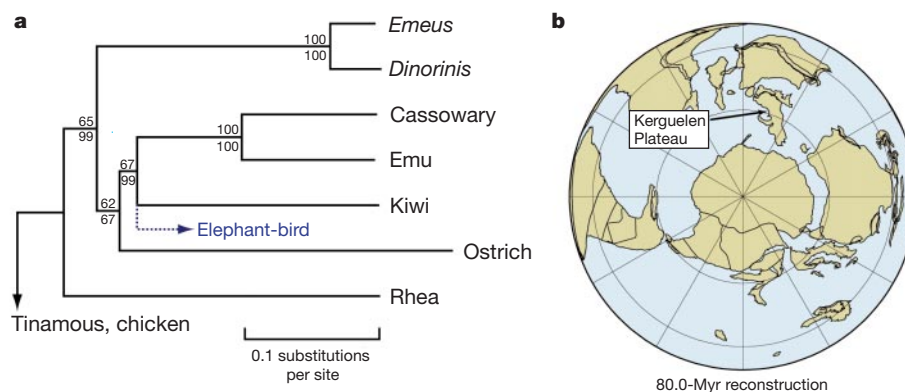


Figure 2 Ratite phylogeography. **a**, Unconstrained maximum-likelihood tree of ratite taxa, rooted with two tinamou and the chicken, using 10,767 bp of mtDNA protein coding sequence. Bootstrap values from 1,000 replications are given above the lines, with nearest-neighbour values⁴ underneath. The maximum-likelihood position of the elephant-bird calculated with the short dataset is indicated, but the data prevent an accurate estimate of branch length. Alternative phylogenetic hypotheses for ratites (New Zealand

ratites as monophyletic outgroup^{1,8} or ostrich as outgroup^{2,6}) both fit the data significantly worse than Fig. 2a ($P < 0.001$). **b**, Tectonic reconstruction of Gondwana at 80 Myr BP. The ostrich (and elephant-bird) are proposed to have crossed the Kerguelen Plateau (indicated) to Indo-Madagascar^{24,25} and eventually Eurasia. Reconstruction map from www.odsn.de/odsn/services/paleomap/paleomap.html.

Table 1 Differences between cloned PCR products and direct sequences of moa, *Mullerornis* and extant taxa

Taxons	Gene	Positions	N	Total (bp)	Ts	Tv	Substitutions		Error per 1,000	Insert (bp)
							Single	Multi		
Extant										
Tataupa	ND1	4,747–5,201 (456)	24	7,308	12	3	15	0	2.05	–
Kiwi	ND4L	11,120–11,532 (390)	11	3,672	13	0	11	1	3.54	–
Cassowary	COIII	10,161–10,743 (593)	16	4,513	10	3	13	0	2.88	–
Extinct										
Oxford										
<i>Dinornis</i>	CR	16,733–00441 (600)	17	10,012	13	0	13	0	1.30	–
	ND1	4,747–5,201 (456)	10	4,560	6	1	7	0	1.54	–
<i>Emeus</i>	COII/ATP6	8,861–9,349 (492)	10	4,310	5	1	6	0	1.39	–
	ND4	11,120–11,598 (478)	10	4,560	7	4	7	2	2.41	–
	16S/ND1	3,787–4,311 (519)	10	5,190	12	0	12	0	2.31	–
	COI	7,807–8,328 (521)	7	3,353	4	0	4	0	1.19	–
	COIII	10,161–10,743 (581)	19	9,411	16	2	18	0	1.91	–
<i>Mullerornis</i>	ND4/ND5	12,788–13,200 (415)	10	3,640	9	0	9	0	2.47	1 (7)
	12S	1,856–2,020 (163)	9	1,467	5	1	6	0	4.08	–
London										
<i>Dinornis</i>	12S	1,753–2,148 (396)	9	3,168	5	0	5	0	1.58	–
	COI	7,807–8,325 (513)	9	4,615	7	1	8	0	1.73	–
	Cytb	15,303–15,783 (479)	5	2,395	5	0	2	1	2.09	–
Barcelona										
<i>Emeus</i>	COII	8,320–8,807 (485)	11	5,335	14	3	13	1	3.19	–

More than 50 kb of moa mtDNA sequences and 15 kb of the extant taxa were cloned and sequenced. Substitutions for the direct sequence were scored as errors, and a rate per 1,000 bp calculated (error per 1,000), ignoring inserts. Positions are numbered relative to the chicken COI sequence. N = number of clones, total bp = total number of positions cloned, Ts = transition, Tv = transversion, single = singleton substitution (appearing only in one clone), multi = substitution common to more than one clone. Insert, insert relative to the direct sequence, with the number of base pairs in parentheses.

Table 2 Estimated divergence dates of the ratite lineages

Calibration	Divergence	Estimated divergence date, with 95% CI (Myr)
Moa-O,E,C,K - 82 Myr	Rhea—other taxa	89.1 (84.4–94.3)
	Moa—O,E,C,K	82
	Ostrich—E,C,K	75.5 (72.5–78.4)
	Kiwi—E,C	68.0 (64.5–71.6)
	Emu—cassowary	35.4 (33.1–38.6)
	<i>Emeus</i> – <i>Dinornis</i>	13.2 (11.9–14.6)
	Estimated evolutionary rate,	0.27% per Myr

Estimated divergence dates, with 95% confidence intervals (CI), using a geological calibration (italics) and 10,767 bp of mtDNA protein coding sequence. Notably, the predicted emu–cassowary split is consistent with palaeontological data. The two-parameter molecular-clock model was used, with the ostrich allowed a separate rate (although the estimated dates are similar whether or not the ostrich is allowed a separate rate estimate). The estimated evolutionary rate per site per Myr is given. O, ostrich; K, kiwi; E, emu; C, cassowary.

Minimal divergence dates for the ratite lineages were estimated using the geological calibration point (Table 2). These molecular estimates predict an emu/cassowary split at 33–39 Myr, encouragingly consistent with the palaeontological data. The concordance between the molecular estimates and the two calibration points give strong support for a Late Cretaceous origin for ratites and, by implication, for other modern avian orders. These divergence dates are compatible with vicariance events during the break-up of Gondwana, apart from the kiwi, for which a subsequent dispersal event is required. The tight clustering of dates and lack of phylogenetic resolution, despite the long sequences, also suggests that the derived ostrich, kiwi and emu/cassowary taxa speciated rapidly, possibly in the remnant Gondwanan landmass of Antarctica/Australia.

The molecular phylogeny in Fig. 2 can be used to provide a temporal framework for biogeographical events during the Late Cretaceous. Thus, the 65–72-Myr divergence of the kiwi provides a provisional estimate for dating limited land-based dispersals between Australia, New Caledonia and New Zealand, presumably along the Norfolk Ridge or Lord Howe Rise, consistent with biogeographic evidence^{17,21,22}. More importantly, the ostrich divergence date of 73–78 Myr (Table 2) clearly post-dates the separation of South America and Africa around 90 Myr, suggesting an alternative biogeographic origin. The earliest ostrich fossils (skeletal and putative eggshell) are from early to mid Tertiary deposits in Europe, India and Mongolia, leading to hypotheses that the ostrich evolved in Eurasia before entering Africa^{7,23}. Reports of Late Cretaceous connections (~80 Myr) between Australia/Antarctica and Indo-Madagascar through the Kerguelen Plateau^{24,25} (Fig. 2b) suggest an alternative hypothesis, more consistent with the molecular phylogeny. Such a land link may have allowed both the elephant-bird and ostrich to enter Indo-Madagascar, with the ostrich eventually arriving in Eurasia via the northerly movement of India. If correct, this hypothesis provides a temporal framework for biogeographic dispersals of other land-based taxa between South America/Antarctica and Eurasia/Africa around this time^{20,24,25}.

By providing a new perspective on Cretaceous biogeography, this ratite molecular phylogeny can be used to discriminate among competing hypotheses about the origin of taxa that arose during the break-up of Gondwana. It also indicates the potential value of using ancient DNA techniques and recently extinct taxa to resolve critical biogeographical issues.

Methods

Ancient DNA studies are extremely susceptible to contamination with extraneous DNA, and must demonstrate adequate experimental and authentication procedures^{11,13,14,26}. Consequently, appropriate negative extraction and amplification controls were used throughout, along with rigorous authentication procedures such as quantitation and cloning^{13,26} (see Supplementary Information). Two divergent moa taxa were sequenced to allow reciprocal authentication, and results were independently replicated in two laboratories. Overlapping regions of sequence matched identically, and different DNA extractions from the same individual always gave identical sequences. The study was

principally performed in Oxford, where all experiments involving ancient specimens before PCR were performed in the Museum of Natural History, a building free of other molecular research.

DNA extraction

DNA was extracted from 0.1-g samples of cortical bone removed from three moa and one elephant-bird specimen: *Emeus crassus*, Museum of New Zealand (MNZ) S91, tibiotarsus from Castle Rocks, Otago; *Dinornis giganteus*, MNZ S34094, phalange from Hodge Ck, Takaka; *Megalapteryx didinus*, MNZ S23808, tibiotarsus from Mt Owen, Takaka; and *Mullerornis agilis*, MNZ S38300, tibiotarsus from Beloha, Madagascar. Accelerator mass spectrometry carbon dating of bone collagen at the Rafter Laboratory, Lower Hutt, New Zealand, showed that the *Emeus* specimen was 1,330 to 1,160 yr BP (95% CI, NZA 9516), and the *Dinornis* specimen was 703 to 523 yr BP (95% CI, NZA 9517). Amino-acid analysis showed that both specimens were moderately/well preserved (Stafford class III), with a C:N ratio of 2.8. The *M. didinus* specimen has been previously dated at 3,350 ± 70 yr BP (see ref. 3). Whole genomic DNA was extracted from tissue samples from the collection of A. Wilson (now held by S. Pääbo, Max Planck Institute for Molecular Anthropology, Leipzig) of an Elegant Crested tinamou (*Eudromia elegans*, breast muscle), Tataupa tinamou (*Crypturellus tataupa*, toe) and cassowary (*C. casuaris*, muscle). Other samples were kiwi (erythrocytes, supplied by M. Potter, Massey University, New Zealand, from specimen K86, Northland, New Zealand) and emu tissue (Louisiana State University Museum of Zoology B-8607, from San Diego Zoo).

PCR and sequencing

PCR amplifications of mtDNA 12S sequences revealed that roughly 600-bp PCR amplifications could be obtained from the *Emeus* and *Dinornis* DNA extracts, compared with 500 bp for *Megalapteryx* and ~200 bp for *Mullerornis*. The moa sequences were identical to previous reports^{3,14}. For efficiency, it was decided to sequence the entire mtDNA genomes of just *Emeus* and *Dinornis*. Subsequently, a series of primer pairs was designed to amplify the entire moa mtDNA genome in 400–600-bp sections, using aligned sequences of the chicken, ostrich and rhea mitochondrial genomes (GenBank accession nos X52392, NC001953, AF090339). The amplified sections overlapped by 4–212 bp (median 44 bp) to produce a contiguous sequence. At least one primer of each pair was designed to be incompatible with human mtDNA sequences¹⁴ to remove the risk of human contamination.

The *Mullerornis* specimen was poorly preserved, and no attempt was made to sequence the entire genome. Instead, sections of the 12S, COII, ATP8 and Cyt *b* genes were amplified in short 150–200-bp sections (Fig. 1) and the resulting sequence (~1000 bp) was compared with the other taxa. Long-range PCR (GeneAmp XL-PCR, Perkin-Elmer) was used to amplify two overlapping regions of 6–8 kb (16S-COIII and COI/tSer-tPro) of the mitochondrial genomes of the kiwi, emu, cassowary and two tinamou taxa to minimize (or detect) the possibility of amplifying numts (Fig. 1). Products were recovered from agarose gels and 400–500-bp regions re-amplified and sequenced using the moa primers. Sequencing reactions were performed on both strands using an ABI BigDye PRISM kit and ABI 377. Sequences were deposited on GenBank, with accession numbers AY016010–016019.

Quantitation and cloning

The amount of moa mtDNA fragments of length 540 bp was determined using an oligonucleotide construct (08334Fquant) and competitive PCR as described¹³ (Fig. 1). To further examine the validity of the direct sequencing results, we carried out PCR amplifications of the four classes of mtDNA genes (rRNA, tRNA, protein coding and control region), and cloned and sequenced the products (Table 1).

Authentication

To replicate the results independently and provide an additional test of authenticity, the extraction, amplification, cloning and direct sequencing experiments were replicated at the University of Barcelona and the Natural History Museum, London, in dedicated ancient DNA laboratories, using separate bone samples and components. Sections of the *Emeus* 12S, 16S and COII genes (Barcelona), *Dinornis* 12S, COI and Cyt *b* genes (London) and *Mullerornis* 12S (London) were sequenced. The *Emeus* COII and *Dinornis* COI, 12S and Cyt *b* products were also cloned (Table 1).

Phylogenetic methods

Maximum-likelihood trees were estimated by both heuristic and constrained exhaustive searches using PAUP 4.0b4a (ref. 27), a general reversible model of substitution and discrete gamma model (see Supplementary Information). Heuristic searches (ten random additions plus branch swapping), and a constrained exhaustive search (where the two moas, the two tinamous and the emu and cassowary were each assumed to form monophyletic pairs and all 105 possible unrooted trees were examined) produced the same maximum-likelihood tree (Fig. 2). Nearest-neighbour interchange values were calculated using bootstrap frequency outputs to determine the local stability of the phylogenetic tree⁴. Alternative phylogenetic hypotheses were tested using a parametric bootstrap²⁸, and were significantly worse ($P < 0.001$) than the maximum-likelihood tree in Fig. 2. The maximum-likelihood position of *Mullerornis* was calculated using the short (1,000-bp) dataset, with the other taxa constrained to their positions in Fig. 2. The resulting placement (Fig. 2) does not have strong support, although no signal linked *Mullerornis* with any ratite taxon in particular (data not shown).

To estimate the divergence dates of the ratite lineages, a molecular clock approach was used. The tree in Fig. 2 was calibrated using the geological divergence estimate of 82 Myr

for the separation of the moa, which was consistent with the estimated emu/cassowary split at 30–35 Myr. The analysis was a simple extension of a described method²⁹ to allow more than four taxa. The assumption of rate constancy among the ratites was tested using a likelihood ratio test of the molecular clock model³⁰. With a likelihood ratio of 12.68, rate constancy can be rejected ($P < 0.01$). However, Fig. 2 suggests that the ostrich may have an elevated rate of substitution, so the test was repeated with the ostrich allowed a different rate from those of other ratites. The resulting likelihood ratio of 0.449 ($P = 0.92$) shows that this two-rate model is consistent with clock-like behaviour. The two-rate model has little effect on the divergence estimates (Table 2), with ostrich dates becoming younger by 5% of the largest change.

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Supplementary information, including clone and primer sequences is available on Nature's World-Wide Web site (<http://www.nature.com>), or on <http://evolve.zoo.ox.ac.uk/data/Ratites/>, or as paper copy from the London editorial office of Nature.

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Wolbachia-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*

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Wolbachia are cytoplasmically inherited bacteria that cause a number of reproductive alterations in insects, including cytoplasmic incompatibility^{1,2}, an incompatibility between sperm and egg that results in loss of sperm chromosomes following fertilization. *Wolbachia* are estimated to infect 15–20% of all insect species³, and also are common in arachnids, isopods and nematodes^{3,4}. Therefore, *Wolbachia*-induced cytoplasmic incompatibility could be an important factor promoting rapid speciation in invertebrates⁵, although this contention is controversial^{6,7}. Here we show that high levels of bidirectional cytoplasmic incompatibility between two closely related species of insects (the parasitic wasps *Nasonia giraulti* and *Nasonia longicornis*) preceded the evolution of other postmating reproductive barriers. The presence of *Wolbachia* severely reduces the frequency of hybrid offspring in interspecies crosses. However, antibiotic curing of the insects results in production of hybrids. Furthermore, F₁ and F₂ hybrids are completely viable and fertile, indicating the absence of F₁ and F₂ hybrid breakdown. Partial interspecific sexual isolation occurs, yet it is asymmetric and incomplete. Our results indicate that *Wolbachia*-induced reproductive isolation occurred in the early stages of speciation in this system, before the evolution of other postmating isolating mechanisms (for example, hybrid inviability and hybrid sterility).

Symbiotic microorganisms are widespread in nature and often have intimate associations with their hosts, ranging from mutualistic to parasitic relationships. It has been suggested that these associations may act as a source of evolutionary innovation for their hosts, leading to differentiation between host populations and ultimately to the evolution of new species⁸. *Wolbachia* are particularly good candidates for symbiont-induced speciation, because these bacteria can modify compatibility between eggs and sperm of hosts, and thus directly cause reproductive isolation without long-term coevolution of the host and symbiont⁵. There is some empirical evidence for a role of *Wolbachia* in speciation in mushroom-feeding *Drosophila*⁹, the flour beetle *Tribolium*¹⁰, and parasitic wasps¹¹. However, the view that *Wolbachia* are involved in invertebrate speciation is still controversial^{5–7}. Here we present evidence that *Wolbachia*-induced reproductive isolation precedes the evolution of other postmating isolating mechanisms in *Nasonia*. The finding supports the view that *Wolbachia* can play a role in reproductive isolation and speciation.

Nasonia is a complex of three closely related species of haplodiploid parasitic wasps. *Nasonia vitripennis* is found worldwide, and is a generalist that parasitizes a variety of fly species. *Nasonia giraulti* occurs in eastern North America and *N. longicornis* in western North America, where they parasitize the pupae of blowflies in birds' nests¹². Genetic and molecular evidence shows that *N. giraulti* and *N. longicornis* are more closely related sister species. Estimates place the divergence of these two species at around 0.250 Myr ago and their divergence from *N. vitripennis* at around 0.800 Myr¹³.

All three species are infected with *Wolbachia*, and individuals of each species are typically infected with two different bacterial types, each belonging to the two major subgroups of arthropod *Wolbachia* (A and B). Furthermore, phylogenetic analysis (data not shown) indicates that the A group bacteria of each species are not closely

related to each other, and therefore have been independently acquired by horizontal transmission. Thus, the *Nasonia* system appears to be prone to the acquisition of *Wolbachia*, and is a promising system for studying the role of these bacteria in reproductive isolation.

Previous studies have shown *Wolbachia*-induced bidirectional incompatibility between two diverged species, *N. vitripennis* and *N. giraulti*¹¹. F₁ hybrids are not formed unless *Wolbachia* are removed by antibiotic curing. However, several other isolating barriers exist between these species, including high levels of F₂ hybrid lethality, abnormal courtship behaviours in F₂ hybrid males (behavioural sterility), and partial premating (sexual) isolation (refs 15, 16, and F.P.O'H., A.C. Chawla and J.H.W., manuscript in preparation). It is therefore unclear whether *Wolbachia*-induced cytoplasmic incompatibility (CI) evolved before the evolution of other isolating barriers or after the divergence of the species. If *Wolbachia* play a causal role in speciation, cases where *Wolbachia*-induced CI evolved before other mechanisms of reproductive isolation should exist.

Here we investigate the role of *Wolbachia* in reproductive incompatibility in a younger species pair, using the more closely related species *N. giraulti* and *N. longicornis*. First, we screened field-collected insects to determine the frequencies of infections in natural populations of the three species. A polymerase chain reaction (PCR) method was employed using previously published specific primers¹⁷. In all three species, 100% of the individuals from various geographical areas were found to be infected (*N. giraulti*, *n* = 29; *N. longicornis*, *n* = 31; *N. vitripennis*, *n* = 31). All samples were doubly infected with A and B, except for one *N. longicornis* strain with a single A infection. Sequence analysis of PCR-amplified products of the *wsp* gene¹⁸ from a subset confirms that the species are infected with species-specific *Wolbachia*, and that the *Wolbachia* from different intraspecific strains form monophyletic groups (data not shown), with little sequence variation within a host species.

We undertook experiments to determine whether *Wolbachia* cause reproductive incompatibility between the 'young' species pair, *N. giraulti* and *N. longicornis*. Wild-type infected strains and antibiotically cured strains derived from those infected strains were crossed in all pairwise combinations. Results show that bidirectional CI occurs between infected *N. giraulti* and *N. longicornis* (Fig. 1). When *Wolbachia* are present, no F₁ hybrid (female) offspring are produced in the *N. giraulti* male × *N. longicornis* female cross and 29.7 ± 2.6 (mean ± s.e., and hereafter) hybrid offspring are produced in the reciprocal *N. longicornis* male × *N. giraulti* female cross. In contrast, crosses using antibiotically cured strains produce 63.9 ± 4.1 hybrid offspring and 82.9 ± 5.1 hybrid offspring, respectively. Thus, presence of *Wolbachia* causes a 100% reduction in F₁ hybrids in one direction and 62.8% reduction in the other direction. In *N. giraulti* and *N. longicornis*, CI results in both a paternal genome loss¹⁹ and offspring lethality (data not shown). These results show that *Wolbachia*-induced CI is a significant component of reproductive incompatibility between *N. giraulti* and *N. longicornis*.

To assess whether *Wolbachia*-induced incompatibility between *N. giraulti* and *N. longicornis* is one of the first incompatibilities to evolve in the divergence of these species, we tested for several other hybrid incompatibilities. Specifically, we investigated (1) interspecific sperm-egg compatibility, (2) inviability and sterility among F₁ hybrid females and (3) inviability and sterility of F₂ hybrid males. Both spermatogenic and behavioural sterility of F₂ males was examined. All the experiments described below were performed with uninfected individuals to exclude the effects of *Wolbachia* on compatibility and viability.

To investigate viability of F₁ females, we compared the number of progeny produced by females mated to intra- and interspecific males. Crosses with uninfected females show that they produce the same number of F₁ progeny whether they mate with males of

their own species or males of the other species (*N. giraulti* female × *N. giraulti* male, 100.2 ± 5.6 versus *N. giraulti* female × *N. longicornis* male, 99.0 ± 4.0; and *N. longicornis* female × *N. longicornis* male, 68.5 ± 4.1 versus *N. longicornis* female × *N. giraulti* male, 78.5 ± 4.8). As only females are hybrids in a haplodiploid insect, we also compared the number of female offspring produced in intra- and interspecific crosses (Fig. 1). There was no reduction in the number of F₁ hybrid females relative to intraspecific controls. Finally, we compared the number of eggs laid during a 6-h oviposition period to the number of adult offspring emerging in hybrid crosses. No significant differences were found (*N. giraulti* female × *N. longicornis* male, 22.5 ± 6.4 eggs versus 20.7 ± 7.1 adults; reciprocal cross, 25.0 ± 9.2 eggs versus 24.3 ± 3.2 adults). Therefore, results clearly indicate that there is no significant F₁ hybrid inviability. They also show that there is no reduction in fertilization of eggs based on whether the sperm came from hetero-specific or homospecific males, indicating no incompatibilities in the fertilization mechanism between these species.

The level of F₁ hybrid female fertility was measured by counting eggs laid by females during a time-limited oviposition period. F₁ hybrid females did not show reduced fertility relative to non-hybrid control females (Fig. 2). In fact, hybrid females with the *N. longicornis* cytoplasm laid significantly more eggs than non-hybrid *N. longicornis* females (Mann-Whitney *U*-test (*U*), *P* < 0.001).

Sterility and/or mortality of F₂ progeny (hybrid breakdown) is one of the earlier manifestations of genetic incompatibility between recently evolved species^{20,21}. This is believed to be due to the general recessivity of genes involved in hybrid inviability and infertility^{20–22}. The haploidy of males in *Nasonia* offers an advantage to the study of recessive incompatibility factors, as such factors will be readily expressed in haploid males^{15,22}. We investigated inviability by comparing the number of F₂ eggs laid by F₁ virgin females to the

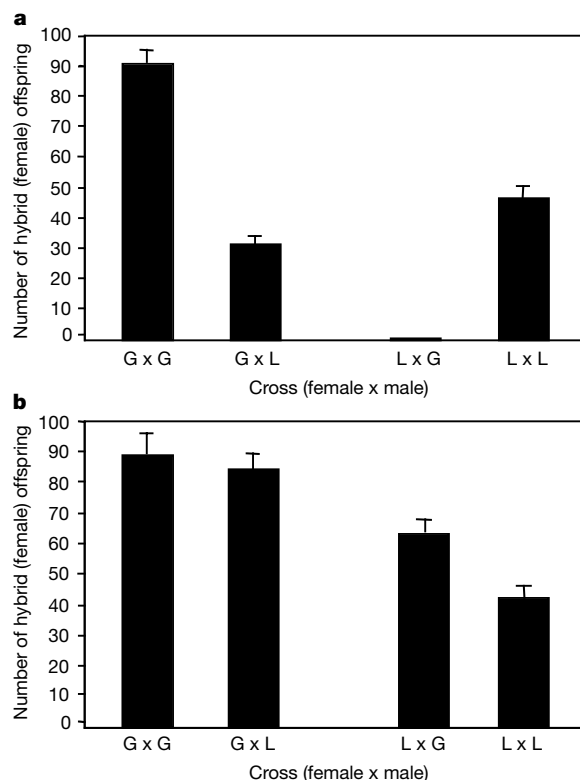


Figure 1 Number of hybrid (female) offspring produced from intra- and interspecific crosses. Results are shown for infected individuals (a) and uninfected individuals (b). Data are the mean number ± s.e. of F₁ progeny. G and L denote *N. giraulti* and *N. longicornis*, respectively.

number of F₂ males that survived to adulthood. (Virgin females produce haploid male progeny from unfertilized eggs in this haplodiploid insect.) There are no significant differences in mortality levels among the F₂ hybrid males relative to the non-hybrid controls (Fig. 2). Mortality was found among F₂ males of hybrid females from the *N. longicornis* male × *N. giraulti* female cross (mean ± s.d. = 19.3 ± 0.4% (ref. 23) mortality; U, egg versus adult number, $p = 0.002$). However, a similar level of mortality was also observed among non-hybrid *N. giraulti* males (F₂ males from the *N. giraulti* male × *N. giraulti* female cross; 14.3 ± 0.3% mortality, $P = 0.009$). No significant differences were found between these crosses in the number of F₂ eggs (U, $P = 0.595$) or F₂ surviving adults (U, $P = 0.862$). Therefore, there is not elevated mortality among hybrids. This finding is quite different from what is found in the older species pair (*N. giraulti* × *N. vitripennis*), which has high levels (70–85%) of F₂ hybrid male mortality¹⁵. Such recessive genetic incompatibilities have apparently not yet evolved between *N. giraulti* and *N. longicornis*.

We assessed the fertility of F₂ hybrid and non-hybrid males by dissecting testes and categorizing sperm motility into three groups: normal, reduced or absent. All males possessed some motile sperm. The percentage of males with normal quantities of motile sperm was 94.7% ($n = 19$) and 95.0% ($n = 20$) for the two hybrid genotypes and 95.0% ($n = 20$) and 100% ($n = 19$) for non-hybrids. Additionally, we tested the ability of hybrid and non-hybrid sperm to fertilize both *N. giraulti* and *N. longicornis* eggs. Of 69 males that copulated, only one failed to produce female offspring, but this occurred in an intraspecific cross. Thus, hybrid sperm is completely functional. This contrasts to many studies in *Drosophila*, which indicate a prevalence of hybrid male sterility loci^{24,25} and that spermiogenic sterility evolves rapidly in the divergence between species^{20,21}.

F₂ hybrid breakdown can also affect courtship behaviour, due to a general 'sickness' of hybrid males or to specific negative interactions in genes involved in courtship behaviour²⁶. We assessed the ability of hybrid and non-hybrid males to (1) locate and mount females, (2) perform the ritualized courtship display, and (3) copulate with females. The type of female did not influence probabilities of initiating courtship and no differences were found among males in their ability to locate and mount females (hybrids, 93.7% ($n = 187$); *N. longicornis*, 97.8% ($n = 46$); *N. giraulti*, 95.7% ($n = 46$); $X^2 = 1.42$, 2 degrees of freedom (d.f.), $P = 0.49$). Among males who successfully mount females, there was a small and nearly significant difference in the proportion of males performing the courtship display (hybrids, 94.2% ($n = 172$); *N. longicornis*, 100% ($n = 45$); *N. giraulti*, 100% ($n = 45$); $X^2 = 5.44$, 2 d.f., $P = 0.07$). Among those males who courted *N. giraulti* females, no differences were found in the proportion of males copulating (hybrids, 91.5% ($n = 94$);

N. longicornis, 95.8% ($n = 24$); *N. giraulti*, 96.0% ($n = 25$); $X^2 = 0.97$, 2 d.f., $P = 0.62$). However, males did differ in their ability to copulate with *N. longicornis* females (hybrids, 52.9% ($n = 68$); *N. longicornis*, 95.2% ($n = 21$); *N. giraulti*, 21.2% ($n = 19$); $X^2 = 22.74$, 2 d.f., $P < 0.001$). This difference cannot be attributed to hybrid breakdown, because hybrid males with *N. longicornis* females copulate at significantly higher rates than do *N. giraulti* males ($X^2 = 6.08$, 1 d.f., $P = 0.014$).

The above results are therefore best explained as mate discrimination of *N. longicornis* females against F₂ hybrid males, rather than to F₂ hybrid 'sickness'. In contrast, our findings with F₂ hybrid males from the older species pair (*N. giraulti* and *N. vitripennis*) indicate high levels of reproductive incompetence throughout the various stages of courtship and mating. For example in the older species cross, 27.6% of F₂ hybrid males failed to locate and mount females, and of those that did mount females, 26.8% failed to perform the ritualized courtship display. As a result, a total of 53.2% of F₂ hybrid males in the older species cross fail to successfully mount females and perform the courtship display (compared to only 13.8% who fail to do so in the younger species cross, not significantly different from controls). We conclude that the genetic incompatibilities responsible for these problems have not arisen since the more recent divergence of *N. giraulti* and *N. longicornis*.

Finally, we investigated the level of premating isolation between the two species in single pair-mating situations. During a 30-min mating period, *N. giraulti* females show no mate discrimination towards *N. longicornis* males, mating at similar frequencies as they do to homospecific males (94.5% mating, $n = 200$ versus 95.6%, $n = 159$, $P = 0.32$). In contrast, *N. longicornis* females show partial mate discrimination towards *N. giraulti* males relative to homospecific males (46.9% mating, $n = 113$ versus 89.9%, $n = 178$, $P < 0.0001$).

The experiments presented here clearly indicate that the species pair *N. giraulti* and *N. longicornis* do not show significant levels of F₁ or F₂ lethality, F₁ or F₂ reproductive sterility, or F₂ 'hybrid sickness' as manifested by competence in courtship behaviour. In contrast, high levels of *Wolbachia*-induced reproductive incompatibility are present in this species pair. Therefore, we conclude that interspecies bidirectional CI has preceded the evolution of these other isolating mechanisms in this system. In addition to *Wolbachia*-induced reproductive incompatibility, there is partial premating isolation in one direction between these species. The strength of premating isolation, at least under the conditions tested here, is weaker than the postmating reproductive incompatibilities caused by *Wolbachia*.

The role of *Wolbachia* in speciation is a matter of current debate^{5–7} and so far, there is limited empirical support for it^{9–11}. We do not claim that *Wolbachia* are currently causing reproductive isolation between *N. giraulti* and *N. longicornis* in nature. Other factors, such as geographical isolation (allopatry) are likely to be more important. However, our results do show that *Wolbachia*-induced bidirectional CI has preceded the evolution of other intrinsic, postmating reproductive isolation barriers in these newly evolving species. The present work therefore provides further support for the argument that the cytoplasmic bacterium *Wolbachia* could promote host speciation. □

Methods

Crosses for assay of CI and copulation frequencies

Single pairs of male and female virgins were observed for 30 min in a 12 × 75-mm vial. We only collected data on incompatibility relationships from crosses with an observed copulation. After 24 h, the male was discarded from the vial, and each mated female was hosted with two *Sarcophaga bullata* blowfly pupal hosts for egg laying. F₁ progeny were scored for sex ratio and family size upon death. RV2, RV2R, IV7 and IV7R2 are the *N. giraulti* and *N. longicornis* infected and uninfected strains, respectively. Uninfected strains were generated from the corresponding infected strains in 1996 through antibiotic treatment of 1% Rifadin (10% sugar water) for three successive generations. Infection status of these strains was confirmed by PCR before the experiments.

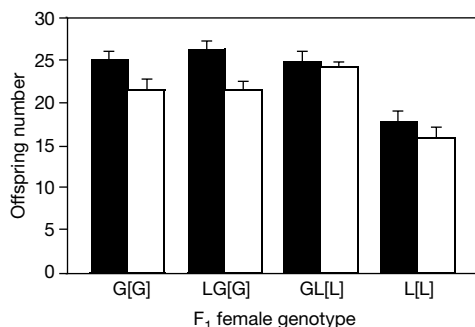


Figure 2 F₂ egg and adult offspring number produced from F₁ hybrid and non-hybrid females. Data are the mean number ± s.e. of eggs (black bar) and surviving adults (white bar). The term in brackets denotes the cytotype, while the term before the brackets denotes nuclear genotype. For instance, LG[G] hybrid females are derived from the cross, L male × G female.

F₂ hybrid viability

F₁ hybrid and non-hybrid virgin adult females (1–2-d old) were placed on four hosts for roughly 48 h for host feeding and egg laying. Females were immediately transferred to one host for a 6-h laying period, after which females were removed from the vial. We limited the ovipositioning period to prevent wasps from becoming resource-limited. Half of these replicates were immediately scored for the number of F₂ eggs laid in 6 h and the remaining half were scored later for the number of adults.

Dissections for sperm motility assay

Testes and seminal vesicles were viewed under a microscope at $\times 400$ magnification for the presence of motile sperm. Tested males were dissected on the day they emerged in a drop of phosphate-buffered saline. At least one testis and one seminal vesicle from each male were viewed. Males were scored as fully fertile if motile sperm were observed in all testes and seminal vesicles observed. Males were scored as partially fertile if a reduced number of motile sperm were observed in any organs viewed.

F₂ hybrid male behavioural and spermiogenic fertility

Single males, aged 18–48 h, were placed in clear 12 $\times$ 75-mm vials with five virgin females, no more than four days old. Behaviour of each male was observed for 15 min. After courtship observation, males were left in the vial with females for an additional 105 min (2 h total) and then removed. Females were then given five hosts for feeding and egg laying. On death of their F₁ progeny, each vial was inspected for the presence of female offspring, indicating successful fertilization of at least one female by the tester male. Behavioural fertility data were not significantly different for F₂ hybrid males from the two reciprocal crosses (F₂ males from *N. giraulti* males $\times$ *N. longicornis* females and from *N. giraulti* females $\times$ *N. longicornis* males), and therefore the data were pooled for statistical analysis.

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Evolutionary radiations and convergences in the structural organization of mammalian brains

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The sizes of mammalian brain components seem to be mostly related to the sizes of the whole brain (and body), suggesting a one-dimensional scale of encephalization^{1–3}. Previous multivariate study of such data concludes that evolutionary selection for enlargement of any one brain part is constrained to selection for a concerted enlargement of the whole brain⁴. However, interactions between structurally related pairs of brain parts⁵ confirm reports of differential change in brain nuclei⁶, and imply mosaic rather than concerted evolution. Here we analyse a large number of variables simultaneously using multi-dimensional methods⁷. We show that the relative proportions of different systems of functionally integrated brain structures vary independently between different mammalian orders, demonstrating separate evolutionary radiations in mammalian brain organization⁸. Within each major order we identify clusters of unrelated species that occupy similar behavioural niches and have convergently evolved similar brain proportions. We conclude that within orders, mosaic brain organization is caused by selective adaptation, whereas between orders it suggests an interplay between selection and constraints.

We use data from the same source^{9,10} as the previous studies^{4,5}. In ref. 4 a small subset of these data was analysed multivariately to study species separations, but in a context where size outweighed most other information; an even smaller subset of the same data was used to study bivariate relationships between pairs of brain parts in ref. 5. Here we examine the complete set of specimen measurements underlying these data by relating the various brain structures in proportion to two reference structures. We explore the detailed structure of the resulting 19-dimensional data space in two stages, and combine the strategies of the previous studies^{4,5} by looking for species relationships as well as associations between variables.

The first stage in this study explores species separations in the subspace spanned by the first three principal components (85% of the information). Figures 1 and 2 show that all orders are clearly differentiated; this shows that they differ uniquely in their internal brain proportions. The three large orders—primates, insectivores and bats—are especially different, being dispersed in nearly orthogonal directions (although not along the orthogonal principal components of the analysis). The primate and insectivore dispersions are separate, but are linked together via some bats. The two

small orders—tree- and elephant-shrews—occupy their own separate locations. Each major order shows some lesser variation in the directions of the greatest dispersions of the others.

The direction along which the primates lie separates the full range of primates from their intersection with the bats at the centre of the overall plot (Fig. 1). This trend differentiates prosimians plus marmosets and tamarins (which are closest to the intersection with the bats) from all cercopithecoid monkeys plus howlers and sakis. These last are differentiated from, in turn, spider and woolly monkeys plus all apes, from which, lastly, humans separate. Hence, in addition to grouping all primates together, this trend tends to differentiate primate species in several broad locomotor categories^{3,11}. It particularly seems to reflect a spectrum of locomotion also apparent in morphometric studies of primate limbs. This spectrum ranges from hind-limb-dominated leaping and squirrel-like scurrying, through quadrupedal (approximately equal limb usage) running and jumping, through forelimb-dominated suspensory climbing and arm-swinging, to human bipedality (which, however, almost certainly evolved from forelimb-dominated arboreal activities)¹².

The insectivore direction separates the various insectivores from the intersection with bats at the centre of the plot (Fig. 1). Within the insectivores the separations proceed from the semiaquatic and burrowing insectivores near the centre, to the surface-dwelling shrews and tenrecs at the periphery. Lesser separations of non-insectivores are also evident in this direction near the centre of the plot: among the microbats the surface-gleaning species¹³, and among the primates, the nocturnal prosimians. Hence, towards the periphery the insectivore direction appears to differentiate species that rely mostly on short-range olfactory and tactile cues when rooting for invertebrate prey in essentially two-dimensional nocturnal niches¹⁴. It separates such insectivorous species from other mammals that are increasingly capable of integrating distance information from various modalities to exploit three-dimensional niches of greater complexity^{2,14}.

The bats, located at right angles (in the data space) to the intersection of primates and insectivores, are arranged with the megabats at one extreme, the phyllostomid microbats intermediately, and the remaining microbats at the other extreme (Fig. 2). The intermediate phyllostomids are a highly diverse group that have radiated from an insectivorous ancestor into a wide range of dietary niches on the South American continent¹³. Their insectivorous representatives lie closer to the other microbats (which are

insectivorous), whereas their frugivorous and nectivorous species converge towards the frugivorous and nectivorous megabats (Fig. 3b). In addition to these major separations among the bats, there are lesser separations in this direction within insectivores, of subterranean burrowers from ground-dwelling or aquatic relatives (Fig. 3a).

Thus, this exploration of the data space demonstrates three very different, largely independent phyletic radiations in the structural organization of the mammalian brain; within each radiation, the placements of species appear to reflect broad spectra of lifestyle variations.

All three major orders include well-documented instances of convergence, in which species belonging to independent lineages known to have evolved separately for between 30–60 million years have come to resemble one another in both body morphology and specialized lifestyle. Similarly, in the neurometric data space we have found that these convergent species tend to lie together, but apart from phylogenetically closer yet less specialized relatives of each, showing that they have also converged in their internal brain proportions (Figs 1 and 3).

Thus, within the primates, the prehensile-tailed spider and woolly monkeys (Atelinae) have independently evolved the same kind of forelimb-dominated climbing–feeding complex that is considered the primary hominoid adaptation¹⁵. Hence, they are regarded as the New World locomotor analogues of the apes¹⁶. Our findings show that the convergence of these monkeys with apes also extends to their internal brain proportions, these particular New World monkeys having surpassed other larger-brained but more quadrupedal Old World monkeys along the primate trend (Fig. 1).

Equally, among the insectivores, different representatives of the ancient, mainly terrestrial, tenrecoid and soricoid lineages have convergently evolved body plans adapted to either aquatic or burrowing lifestyles^{2,10}. Our findings show that these lifestyle groups also converge in their brain proportions (Fig. 3a).

However, it is perhaps among the bats that our findings are especially clear. Fruit- and nectar-eating lifestyles are known to have evolved independently in Old World megabats and in several subfamilies of New World microbats (Phyllostomidae). The specialized frugivorous and nectivorous microbat subfamilies converge in their brain proportions with the frugivorous (but much larger-brain) megabats. Yet their close insectivorous relatives are separate, being associated with the other insectivorous microbats (Fig. 3b).

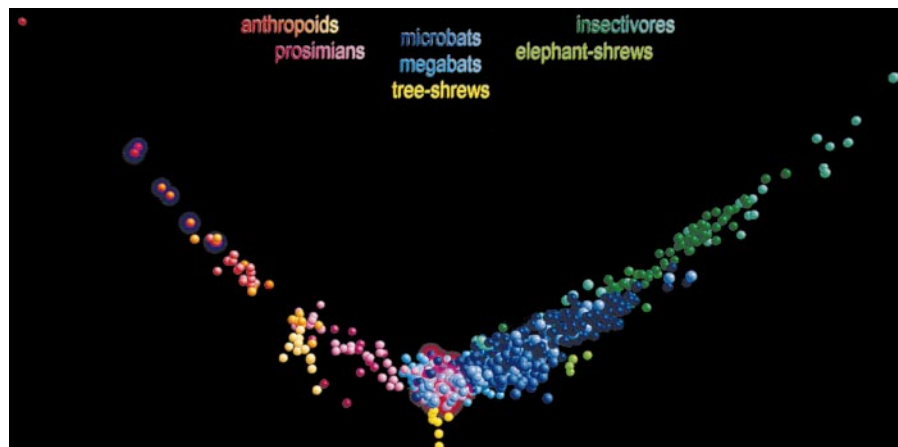


Figure 1 Three-dimensional representation of species (coloured spheres) as separated by the first three Principal Components of the analysis of brain proportions. Spheres are in perspective to show depth. Proximity between spheres reflects similarity between species in brain proportions. The main colour ranges (see key in image) indicate the three main orders: reds through oranges show primates, blues show bats and greens show

insectivores. These orders are dispersed at approximately right angles, indicating independent radiations in brain organization. They do not lie along a single axis, as would be expected if size were the overriding factor. Highlights distinguish lifestyle groupings: violet shows suspensory primates and red shows carnivorous bats.

Furthermore, despite the fact that plant-visiting microbats are thought to be limited to the New World¹³, Fig. 3b shows that the murinine Old World microbats group in parallel to them, implying that they, too, visit plants. Unpublished field observations made subsequent to this study indicate that some murinine bats are indeed nectivorous, an elegant external test of this prediction. Finally, several Old and New World microbat species have independently evolved carnivorous lifestyles¹⁷. These species cluster tightly in the brain data space (Figs 1 and 3b) together with other small relatives sharing the hunting strategy of gleaning prey from ground, vegetation, or water surface using passive listening, vision and broadband echolocation¹³.

The second stage of this study realigns the principal component axes along these three major directions through standard rotation, and this permits us to display the clusters of brain proportions in each direction as they perform the species separations.

Thus Fig. 4a shows that the dispersion of species in the primate direction is produced by a concerted increase in the proportions, relative to the medulla, of neocortex, striatum, cerebellum and diencephalon. Almost all regions of the neocortex project to the (dorsal) striatum and (lateral) cerebellum which, via the thalamus in the diencephalon, project back to the cortical motor, premotor and prefrontal areas^{18,19}. Hence, these brain parts are tightly integrated into a distributed system that contains the highest levels of the hierarchy of voluntary motor control¹⁸. The primate trend, therefore, may be thought of as comprising an expansion of the higher, rather than the lower, levels of the motor hierarchy (as distributed over medulla and spinal cord)²⁰. Accordingly, it may reflect an increase in voluntary over more stereotyped control of behaviour. This would match the spectrum of complexity and

flexibility of locomotion represented by the separation of species along this trend.

Figure 4a and b also shows that the dispersion of species in the insectivore direction coincides with a cluster of variables consisting of the proportions, relative to the neocortex, of hippocampus, septum, striatum, palaeocortex, and, more obliquely, schizocortex, diencephalon and olfactory bulb. The septum and hippocampus, together with schizocortex and structures in the diencephalon, are closely interconnected²¹ into a functionally integrated septohippocampal system involved in establishing the spatiotemporal location of desired goals²². This system is connected via the amygdala (here included in palaeocortex) with the (ventral) striatum²¹ to form a proposed mesotelencephalic "goal attainment" system²². Hence, proceeding for the conservative tenrecs and shrews at its periphery, this direction can be thought of as representing an expansion of the neocortex relative to this mesotelencephalic system, accompanied by an expansion of midbrain relative to medulla (Fig. 4). Because this direction is orthogonal to the separate expansion of neocortex and associated motor structures along the primate trend, and because the midbrain contains prominent visual and auditory structures, it could represent the extension or addition of cortical sensory projection fields²³. This would suggest an elaboration of the neural ability to integrate multimodal distance information into perceptual representations of increasingly complex niches¹, and this would match the previously observed separation of taxa along this direction.

Figure 4b also presents the cluster of variables associated with the dispersion of bat species from micro- to megabats. It shows that this direction involves an expansion, relative to the medulla, of the same

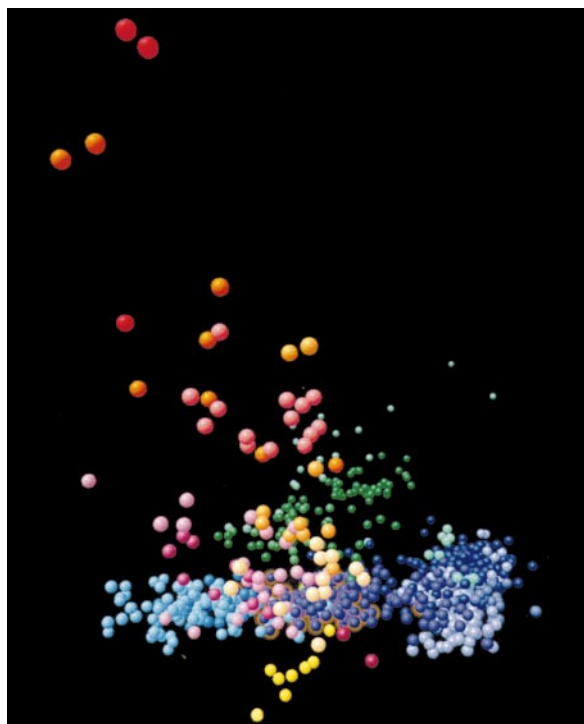


Figure 2 Side view of Fig. 1, showing the bat dispersion at right angles to both the primate and insectivore directions. Colour labelling is the same as in Fig. 1. The bats (blue spheres) are dispersed along the third dimension, while the primates (reds) come up towards the viewer, and the insectivores (greens) recede towards the background. Hence, the full three-dimensional structure of the data somewhat resembles a bird or insect in flight, with the torso formed by the bats, and the two wings by the primates and insectivores, respectively. The bat direction separates the megabats (sky-blue) from the microbats (indigo blue).

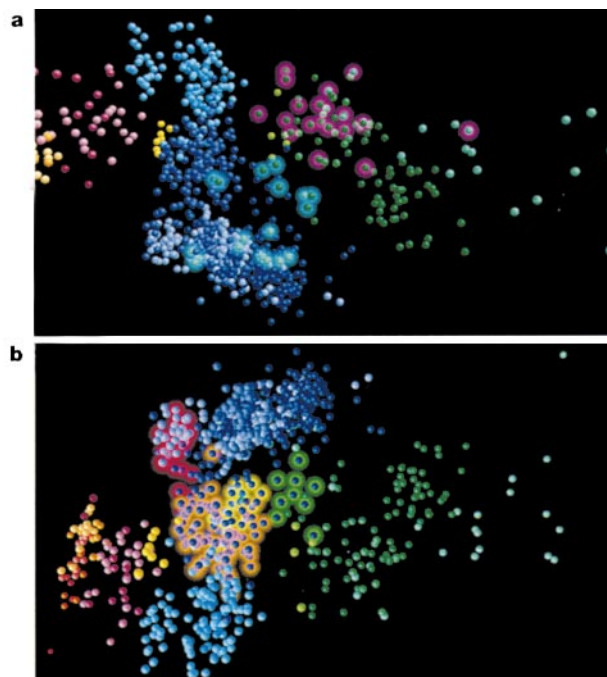


Figure 3 Lifestyle convergences in brain proportions in insectivores and bats. **a**, Separate clusters of the semi-aquatic (turquoise highlights) and fossorial (purple highlights) insectivores, each comprised of convergently evolved representatives of the ancient tenrecoid and soricoid radiations (pale/dark green spheres, respectively). **b**, Several lifestyle convergences within the bats: the frugivorous/nectivorous phyllostomid microbats (orange/yellow highlights) segregate from their insectivorous and carnivorous relatives to converge with the megabats (sky-blue). The murine microbats (green highlights) approach the nectivorous phyllostomids (yellow highlights), suggesting a similar nectivorous lifestyle (R. B. Coles, C. I. Clague, O. J. Whybird and H. J. Spencer, personal communication). Carnivorous microbats (red highlights) of separate evolutionary lineages have converged closely in their brain proportions. Conventions as in Fig. 1.

septohippocampal limbic structures as featured above, but this time associated with a similar expansion of olfactory bulb and palaeo-cortex. This suggests an elaboration of the ability to form spatio-temporal representations of the location of desired goals²² based on olfactory information in frugivorous bats, which rely on an unevenly distributed and ephemeral, odorous food source¹³.

Hence, these rotated axes allow us to recognize clusters of variables representing concerted changes in the relative proportions of different distributed systems of functionally interacting brain parts. They appear to represent essentially separate functional reorganizations of the mammalian brain. When raw data on brain sizes (logarithm of the values) are analysed, their first principal component accounts for 96% of their total variance, and this has been interpreted to mean that their complete information content is affected only by brain size⁴. In contrast, in the analyses of brain proportions, the mean variance for brain size (logarithm of the values) accounts for only 78, 29 and 24% of the variance along the primate, insectivore and bat axes, respectively. It seems clear, therefore, that these internal reorganizations are not merely size-related. Moreover, such associations as exist with either absolute or relative brain size are not related to a single size axis. Increases in different sets of functionally related brain parts occur along different axes in different groups. For example, although in both bats and primates fruit-eating species tend to be more highly encephalized than insectivorous species², our findings indicate that this is owing

to the proportional expansion of different brain subsystems in each.

We have thus identified three independent evolutionary radiations in the structural proportions of the mammalian brain that mirror not only the phylogenetic clusters of species examined, but also their broad behavioural abilities and lifestyles. Second, across these mammals we have documented several independent cases of convergent evolution in the structural proportions of the mammalian brain. Third, we have demonstrated that these various radiations and convergences are associated with changes in the proportional sizes of different functionally integrated sets of brain structures. These findings provide more detailed evidence of mosaic evolution in brain organization⁵, and rule out an overriding influence of uniform developmental constraints on mammalian brain evolution⁴. Thus, findings of independent radiations in brain organization between orders imply that either such constraints are less powerful than we assumed and have been overridden by new selective pressures in the different lineages, or that the developmental constraints have themselves evolved differently since the separations of the original stocks, or both. Within orders, convergences of species with similar lifestyles provide detailed evidence of where and how natural selection has shaped the internal organization of mammalian brains to specific eco-ethological niches. Indeed, this selection appears to have been so pervasive as to allow us to infer important ecological and behavioural attributes of a mammal from multivariate knowledge of its brain proportions

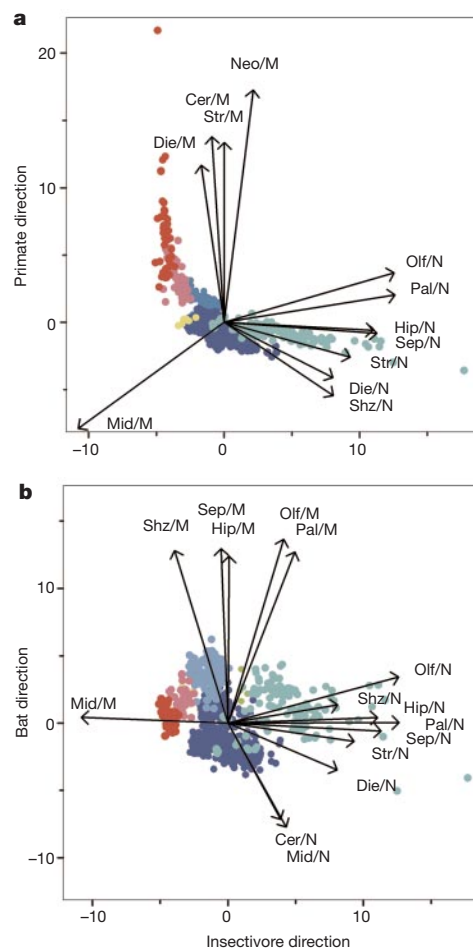


Figure 4 Relative contributions of the various brain structure proportions (arrows) to the species dispersions (dots). Primates are shown in red, insectivores in green and bats in blue. Biplots (two-dimensional) are shown of the rotated components corresponding to **a**, the primate and insectivore, and **b**, the insectivore and bat directions (see Methods). Three clusters of variables are apparent, each associated with one of the major

dispersions in the data. The primate direction coincides primarily with the medullary proportions (M) of cerebellum (cer), striatum (str), diencephalon (die) and neocortex (neo); the insectivore direction with the neocortical proportions (N) of septum (sep), hippocampus (hip), palaeocortex (pal) and striatum; and the bat direction with the medullary proportions of septum, hippocampus and schizocortex (shz).

alone. Hence, these findings not only substantiate the fact of mosaic brain evolution, but begin to unravel its various causes. Finally, the observed evolutionary radiations in brain organization appear to coincide with such fundamental dimensions of cognition as motor planning, perceptual representation, and spatio-temporal memory. Hence, rather than a single evolutionary progression in general 'intelligence' across mammals, different dimensions of 'intelligence' appear to have evolved independently in different lineages. □

Methods

We introduce here a hypothesis-free multivariate morphometric approach¹² to the comparative study of quantitative data on the mammalian brain. Rather than using inferential statistics to test *a priori* hypotheses about the data, we explore the multi-dimensional structure of the data in all its intricate detail using descriptive multivariate statistics²⁴ and data visualization²⁵. Hence it is robust to phylogenetic dependencies between specimens.

We apply this approach to the full complement (921 specimens) of Stephan's original measurements on the volumes of the 11 major divisions of the brain stem and forebrain in 363 species of primates, insectivores, bats, tree-shrews and elephant-shrews. The data include the raw volumes of: medulla (+ reticular formation), cerebellum (+ brachium, nuclei pontis), midbrain (– reticular nucleus), diencephalon, olfactory bulb, palaeocortex (+ amygdala), septum, hippocampus, schizocortex (entorhinal, perirhinal and presubicular cortices), and neocortex (isocortical grey + underlying white matter)¹⁰.

In order to obtain measures that are more sensitive to variations in the functional, systemic interdependence of these brain parts, we reorganized the data within each specimen to reflect variations in major input–output proportions²⁶. We identified two fundamental sets of such projections that vary between taxa: peripheral projections associated with varying body size, and, thus to some extent with the size of the medulla²⁷, and internal projections associated with variations in the size of the neocortex^{5,26}. The size of each brain part was therefore described relative to medulla and neocortex within the same specimen. This resulted in 19 different brain structure proportions across different developmental growth fields²⁶. Although these proportions were derived mathematically, they in fact represent empirically measurable properties that are intrinsic to each specimen.

After univariate and bivariate examination of both the raw and the transformed data sets, a principal components analysis was applied to the correlation matrix of the functionally rearranged brain data. Only the first three components were retained for further analysis, reducing the dimensionality of the data space from 19 to 3, while preserving 85% of its total variance. Phylogenetic and lifestyle associations among species, within the data subspace spanned by these first three principal components, were investigated interactively by dynamic three-dimensional computer representations with data points rendered as colour-coded spheres with coloured highlights (Figs 1 to 3). Varimax rotation aligned these components with the directions of major dispersion for the three largest mammalian orders. This allowed identification through biplots (Fig. 4) of the associations between the variables that contributed most to those dispersions. Biplots combine graphical displays of the relationships between data points and those between variables into a single plot²⁴, thus showing the interrelationships between the two.

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Neurons derived from radial glial cells establish radial units in neocortex

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The neocortex of the adult brain consists of neurons and glia that are generated by precursor cells of the embryonic ventricular zone. In general, glia are generated after neurons during development¹, but radial glia are an exception to this rule. Radial glia are generated before neurogenesis and guide neuronal migration². Radial glia are mitotically active throughout neurogenesis³, and disappear or become astrocytes when neuronal migration is complete^{4,5}. Although the lineage relationships of cortical neurons and glia have been explored^{6,7}, the clonal relationship of radial glia to other cortical cells remains unknown. It has been suggested that radial glia may be neuronal precursors^{5,8–10}, but this has not been demonstrated *in vivo*. We have used a retroviral vector encoding enhanced green fluorescent protein to label precursor cells *in vivo* and have examined clones 1–3 days later using morphological, immunohistochemical and electrophysiological techniques. Here we show that clones consist of mitotic radial glia and postmitotic neurons, and that neurons migrate along clonally related radial glia. Time-lapse images show that proliferative radial glia generate neurons. Our results support the concept that a lineage relationship between neurons and proliferative radial glia may underlie the radial organization of neocortex.

Neurons in mammalian neocortex are aligned in columnar or radial units that receive similar inputs and serve similar functions¹¹. The 'radial unit hypothesis' of neocortical development proposes that functional radial units are established by the proliferation of precursor cells in the embryonic ventricular zone, giving rise to related neurons that migrate into the cortex along shared radial glial fibre guides¹². To test this hypothesis, we made intraventricular injections of green fluorescent protein (GFP)-expressing retrovirus to mark cortical precursor cells and their clonal progeny.

We found that viral infection *in utero* at embryonic day 15 (E15) and E16 (42 embryos) resulted 1–3 d later in radial clusters of cells with common morphological features, suggesting that they are

a repeatedly occurring clonally related cell group. At 24 h after infection, labelled cells were predominantly single, and either resembled radial glial cells with a cell body in the ventricular zone (VZ) or were multipolar or bipolar cells with cell bodies located elsewhere. Some clusters at 24 h after infection comprised two cells: one radial glia-like, and another contacting the radial process. All radial glia-like cells had the defining morphological characteristics of radial glial cells^{4,5}: a nucleus in the VZ; a short process extending to the ventricular surface with a large end-foot; a fine radial process extending to the pial surface; and frequent contact with blood vessels (Fig. 1A).

The retrovirus used randomly integrates into one daughter cell after infection. Asymmetric neurogenic divisions, which predominate at E15–16, generate one neuron per cell cycle. Thus, we would predict that one division after infection, 50% of labelled cells would be progenitors and 50% would be postmitotic neurons. When all GFP-labelled cells in the sensorimotor cortex of a single embryo were examined at 24 h after infection, 49.8% (485/973) were radial glia-like cells with cell bodies located in the VZ (Fig. 1A, 24 h). The remaining cells (488/973, 50.2%) were non-radial glia-like and were found predominantly outside the VZ (469/488, 96%). This

suggested that these two populations of cells might be the products of asymmetric cell divisions from a precursor population of mitotic radial glial cells in the VZ; therefore, we next examined GFP-labelled clones at longer survival times.

At 48 h after infection, we observed radially arrayed clones of cells rather than single radial glia-like cells (Fig. 1A, 48 h). These clones comprised a radial glia-like cell in the VZ, with 1–3 cells arrayed along the radial glial fibre ($n = 32$ clones). At 72 h after infection, typical clones ($n = 111$) consisted of 4.77 ± 0.23 cells distributed along the radial fibre (Fig. 1A, 72 h). Each clone had 1–3 cells in the VZ (average 1.96 ± 0.10), at least one of which was radial glia-like. Most radial clones had one radial glia-like cell, but a minority had two. All other cells in each clone contacted the radial process of the radial glia-like cell. This was a constant feature that allowed us to define radial clones for quantitative analysis. There were 1–2 cells (average 1.52 ± 0.09) in the subventricular zone (SVZ) that were typically multipolar (Fig. 1A). There was usually 1 cell (average 0.97 ± 0.11) in the intermediate zone that sometimes had the appearance of a migrating neuron, with a leading and trailing process aligned along the GFP-labelled radial fibre (Fig. 1A).

Cells in the cortical plate averaged 0.32 ± 0.07 per clone, were

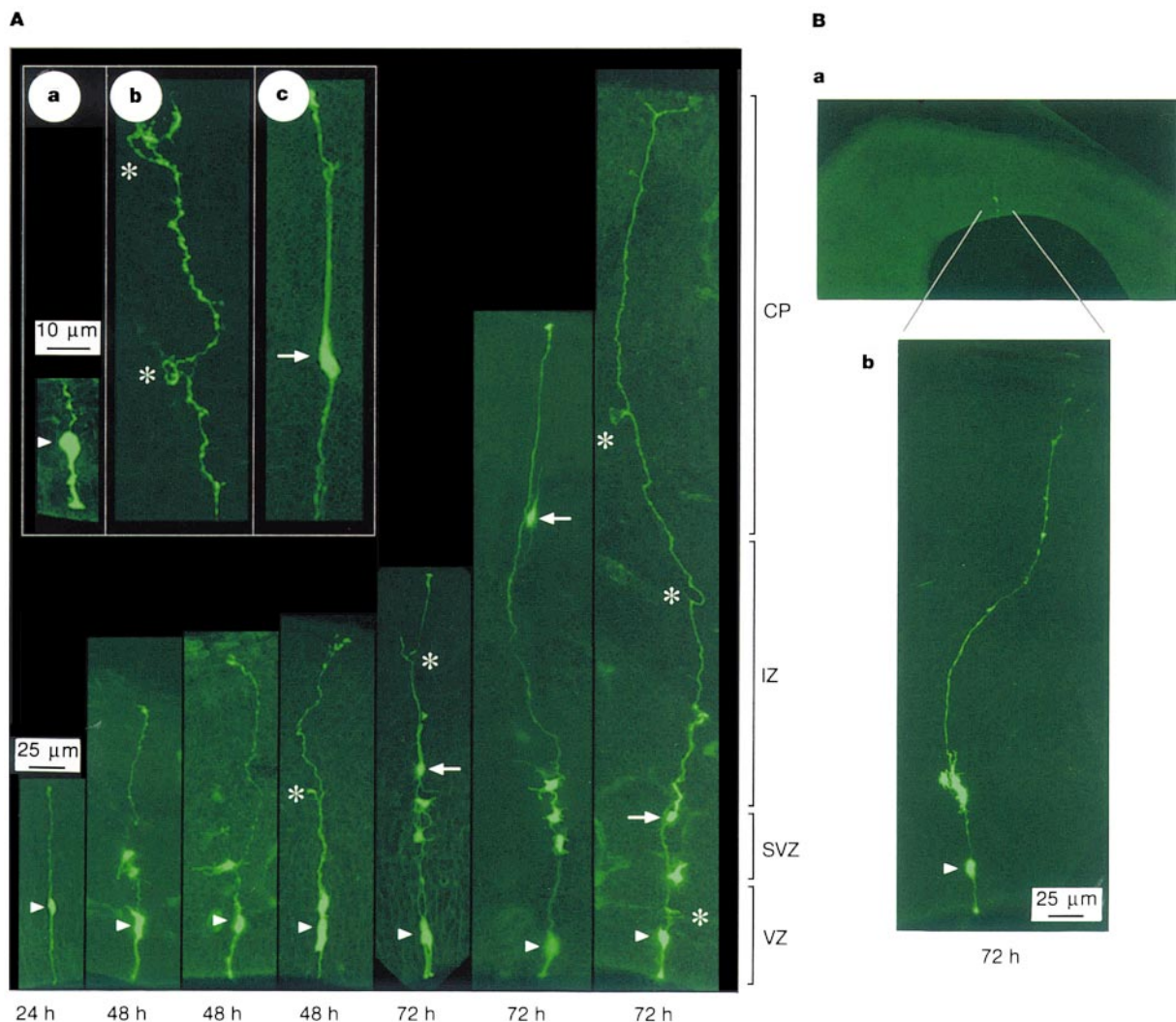


Figure 1 Radial clonal units 24–72 h after retroviral infection. **A**, Each clone has characteristic features: one cell resembles a bipolar radial glial cell (arrowheads) that contacts both pial and ventricular surfaces, and 1–4 additional cells are distributed along the radial process. Cells resembling migrating neurons are often opposed to the clonally related radial process (arrows). Radial processes often contact or encircle blood vessels (asterisk). **a**, Typical appearance of radial glia-like cell body. **b**, Characteristic association

of radial fibres with blood vessels (asterisk). **c**, Presumptive migrating neuron (arrow) associated with a related radial fibre. Indicated cortical plate and intermediate zone boundaries apply to the 72-h clone at far right; boundaries of SVZ apply to 48-h and 72-h clones; and VZ boundaries apply to all. **B**, Dilute viral titres (1:100) produce lower infection rates (**a**), but clones have the same characteristics as above (**b**). CP, cortical plate; IZ, intermediate zone.

sometimes displaced one or two cell diameters from the radial process, and usually resembled pyramidal neurons with rudimentary apical and basal dendrites. Cells resembling neurons migrating along GFP-labelled radial fibres were also found in the cortical plate (Fig. 1A, a). These morphological features suggest that individual clones contain cells of mixed identity, including radial glial cells as well as immature neurons, and support the concept that migrating neurons use clonally related radial glia as guides during migration.

At all ages examined, there were individual scattered cells that had no obvious association with other labelled cells. The largest number of scattered cells occurred 3 d after infection, when 71% (1037/1448, $n = 5$ animals) of GFP-labelled cells were not associated with identified clones. Of these, 4.1% were in the VZ, 48.0% were in the SVZ/intermediate zone and 47.9% were in the cortical plate. These cells most probably reflect the same population of neuron-like cells observed at 24 h, but some may represent tangentially migrating neurons that produce clonal dispersion⁷.

The finding of individual scattered cells suggested that the radial clones might, by chance, contain cells derived from different clones. To eliminate this possibility, we injected three embryos (E15) with inoculate diluted 1:100. This led to a significant reduction in labelled clones, such that examination of serial sections through the cerebral hemispheres at 72 h identified a mean of 3.6 radial clones in the sensorimotor cortex of both hemispheres. In these experiments, 54% of GFP-labelled cells were part of radial clones, and the remaining cells were isolated. Many radial clones were found in sections that contained no scattered cells (Fig. 1B). The radial clones had the same morphological features as observed in embryos receiving higher titres of virus and displaying higher

infection rates. Each clone comprised one radial glia-like cell, and a small cluster of additional cells (3.7 ± 0.52 cells per clone), all in contact with the radial process (Fig. 1B). It is therefore unlikely that we are mistakenly combining separate glial and neuronal clones.

To confirm that clones included both neurons and glia, we used immunolabelling with cell-type-specific markers. At 24 h, $80.5 \pm 10.0\%$ of radial glia-like cells labelled with the GFP retrovirus were positive for vimentin, an intermediate filament protein expressed by radial glial cells¹³ ($n = 3$ embryos, Fig. 2a). At 72 h, $88 \pm 4.6\%$ of radial clones had at least one vimentin-positive cell in the VZ ($n = 5$ embryos); $85.7 \pm 13.9\%$ of these vimentin-positive clones contained one vimentin-positive VZ cell, whereas $14.3 \pm 3.9\%$ had two (Fig. 2b). Vimentin staining could be visualized throughout the GFP-positive radial glial cells, including their radial processes extending to the pia (Fig. 2b). These experiments confirm that radial glia-like cells, a common feature of the GFP-labelled radial clones, are in fact radial glial cells.

Radial glial cells are mitotically active during the period of neurogenesis and undergo interkinetic nuclear migration³, a property associated with neuronal precursor cells. Because clones comprised radial glia at 24 h, and radial glia plus a heterogeneous group of cells at longer survival times, we reasoned that the GFP-labelled radial glial cells were mitotically active. We therefore examined clones for cells expressing the intermediate filament protein, nestin, which identifies central nervous system (CNS) stem cells¹⁴. Nestin is expressed by proliferative neuroepithelial cells, including neuronal precursors, and should be expressed by mitotically active GFP-labelled cells. We found that $73.9 \pm 2.8\%$ ($n = 23$) of GFP-expressing

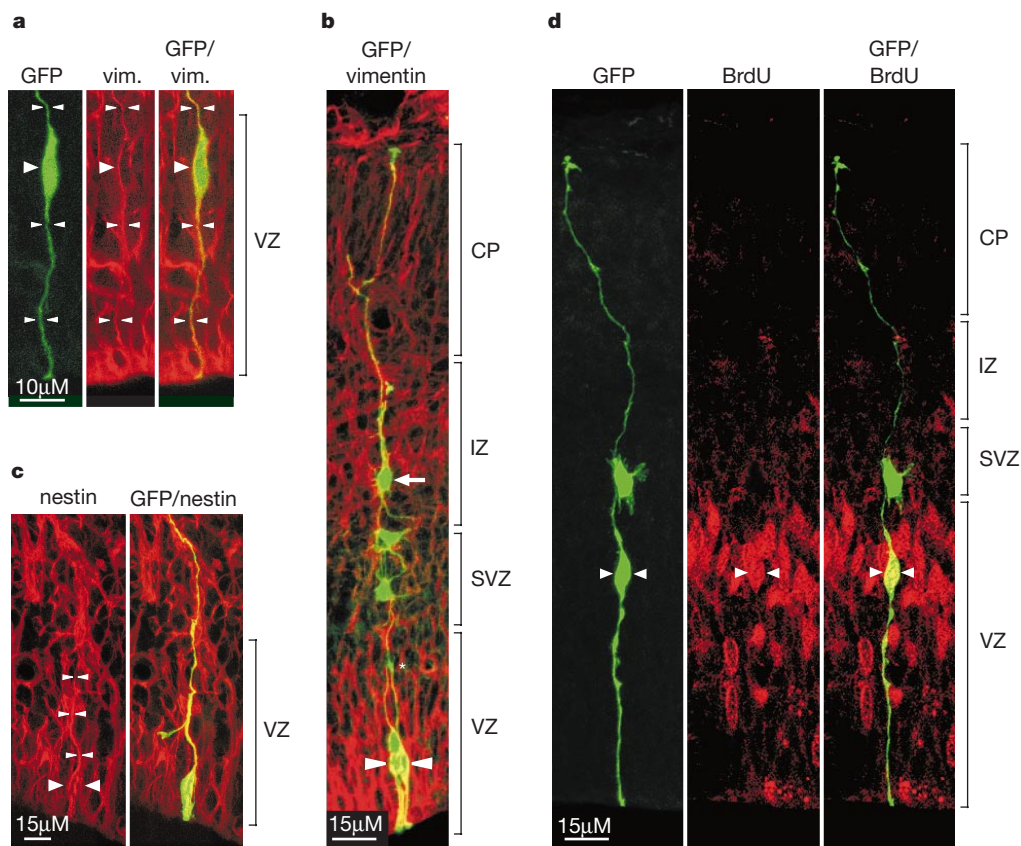


Figure 2 Radial clones contain mitotic radial glial cells. **a**, At 24 h after infection, GFP-expressing VZ cells are positive for the radial glial marker, vimentin (vim.), visible in the perinuclear cytoplasm and in the radial processes (arrowheads). **b**, At 72 h, radial clones include vimentin-positive radial glial cells (large arrowheads). A vimentin-negative cell resembling a migrating neuron (arrow) is apposed to the vimentin-positive radial fibre.

c, Clonal cells in the VZ express the stem-cell marker, nestin (large arrowheads). These cells have nestin-positive radial processes (small arrowheads) extending to the pia (out of view). **d**, S-phase clonal cells labelled with BrdU are radial glia. CP, cortical plate; IZ, intermediate zone.

clones had nestin-positive radial glial cells in the VZ (Fig. 2c). The incorporation of retrovirus by radial glial cells, as well as their expression of nestin, supports the concept that the radial glial cells in labelled clones are mitotically active. However, other cells within clones, for example the SVZ cells, might potentially be proliferative and capable of generating additional clonal cells.

To identify further the mitotically active clonal cell members, we labelled S-phase cells with bromodeoxyuridine (BrdU) *in utero* at 30, 36, 42 or 48 h after viral infection. One hundred per cent of GFP-positive clone members that double-labelled with BrdU had radial glial morphology (Fig. 2d, $n = 82$ clones containing BrdU-positive cells). Because radial glial cells are the only GFP-positive cells that are labelled with BrdU, the radial glial cells appear to be the only cells in the clones capable of being progenitors.

Radial clones included cells with morphological features of immature neurons. We confirmed neuronal identity of these cells using the neuron-specific marker, TUJ1. An example of a multipolar SVZ cell in a radial clone that expresses TUJ1 is shown in Fig. 3A, panel a. Radial clones also contained cortical plate cells that resembled pyramidal neurons. As expected, these cells were also TUJ1 positive (Fig. 3A, b). A characteristic feature of radial clones is the distribution of cells at several levels along the radial glial fibre. To investigate the distribution of neurons within clones, we performed a laminar analysis of the number of GFP-expressing clonal cells labelled with TUJ1 antibody; $23.8 \pm 9.5\%$ of clones contained a TUJ1-positive cell in the SVZ, whereas $30.9 \pm 12.1\%$ of clones contained a TUJ1-positive cell in the intermediate zone. For clones containing cells in the cortical plate, $94.1 \pm 3.3\%$ of these clones contained a TUJ1-positive cell in the cortical plate (Fig. 3A, c). This may reflect a radial gradient of neuronal differentiation in the clones. The results of immunolabelling experiments thus confirm that radial clones contain cells of mixed identity, including at least one proliferative radial glial cell, and one or more neurons.

By using GFP as a clonal marker we could identify clonally related cells in living brain slices, and could therefore characterize their physiological properties. Electrophysiological recordings from radial glia-like cells in the VZ ($n = 11$, injected E15 and E16, recorded E18 and E19) showed membrane properties previously associated with precursor cells¹⁵, including relatively low input resistance, suggestive of electrical coupling to other cells¹⁵, and a lack of voltage-gated sodium conductance, consistent with a non-

neuronal identity (Fig. 3B, a and c). The earliest physiological indicators of postmitotic neuronal identity are high electrical input resistance caused by uncoupling¹⁶ and the development of active membrane conductances. As expected for cells undergoing asymmetrical divisions, we found clear differences in input resistance when we recorded from radial glial cells and presumptive migrating daughter cells ($154 \pm 24 \text{ M}\Omega$ and $531 \pm 34 \text{ M}\Omega$, respectively; $n = 13$).

Recordings from GFP-expressing cells located in the SVZ ($n = 8$) revealed cells with higher input resistance than VZ cells, $1.03 \pm 0.18 \text{ G}\Omega$, and included cells ($n = 4$) with voltage-dependent conductances consistent with immature neurons (Fig. 3B, b and c). As expected, recordings from pyramidal-shaped clonal cells in the cortical plate ($n = 4$) showed high input resistance, $1.43 \pm 0.21 \text{ G}\Omega$, and larger voltage-dependent inward and outward currents (Fig. 3B, b and c). Together, the electrophysiological properties of radial clones and the results of TUJ1, BrdU and nestin immunostaining establish that individual clones include mitotically active radial glia and neurons at varying stages of maturation.

We used whole-cell recording techniques and GFP-labelled clones to introduce intracellular markers into individual cells within clones. Using living brain slices, we filled GFP-labelled cells in the VZ ($n = 13$), intermediate zone ($n = 8$) and cortical plate ($n = 4$) with the fluorescent gap-junction-permeable marker, Alexa 594 (Fig. 3B). In radial glial cells ($n = 13$) the dye spread within the radial process, confirming that other clonally related GFP-expressing cells, whether located in the VZ, intermediate zone or cortical plate, were distributed along the fibre originating from the radial glial cell.

We also found that GFP-expressing cells not located in the VZ were not dye-coupled to the radial glial cell or to other cells in the clone (Fig. 3B, a). We were able to examine whether the relatively low membrane resistance of clonal radial glial cells represented gap-junction coupling, which has been described in cortical precursor cells¹⁵. Using intact cortical slabs, we filled GFP-expressing radial glial cells with the gap-junction-permeable dyes neurobiotin or Alexa 594 ($n = 5$). The dyes filled small clusters of VZ cells, confirming that radial glial cells are coupled to other cells in the VZ. Some clones had two cells in the VZ, and, in two of these cases, both cells were filled with dye after injection of dye into one cell (Fig. 3B, d). These results suggest that some clonally related VZ cells may be coupled to one another.

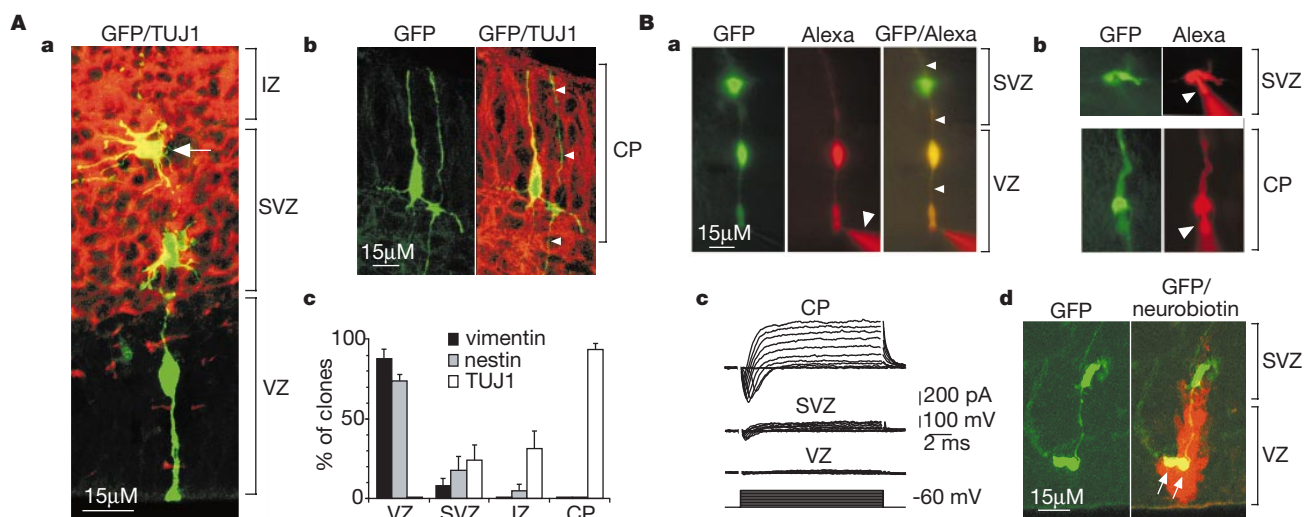


Figure 3 Radial clones contain radial glia and immature neurons. **A, a**, At 72 h, this clone contains one radial glial cell and one cell in the SVZ that expresses the neuronal marker TUJ1 (arrow). **b**, Clonal cells in the cortical plate also express TUJ1. This neuron is adjacent to its radial glial fibre (arrowheads). **c**, Histogram showing the laminar distribution of immunopositive cells in radial clones at 72 h (see text). **B, a, b**, Electrodes

(large arrowheads) were used to fill clonal cells with Alexa 594 (red). **c**, Voltage-dependent currents in clonal cells show progressive development from the VZ to cortical plate. **d**, Filling with neurobiotin (red) shows that clonally related cells in the VZ (yellow, arrows) can be dye-coupled. CP, cortical plate; IZ, intermediate zone.

We next observed individual GFP-expressing radial glia for up to 60 h using time-lapse videomicroscopy of slice cultures from embryos infected *in utero*. As shown in Fig. 1, most cells expressing GFP in the VZ at 24 h after infection are radial glia. Radial glia were identified in time-lapse experiments by morphological criteria, including radial process contact with both ventricular and pial surfaces (Fig. 4B, a). We observed radial glia undergoing interkinetic nuclear migration and dividing at the ventricular surface ($n = 14$, Fig. 4A). Newly born cells migrated along the radial glial fibre in the direction of the cortical plate ($n = 14/14$, Fig. 4A), a behaviour associated with newborn neurons^{2,17}. Several of the cells that migrated out of the VZ were confirmed to be neurons by TUJ1 labelling ($n = 4/5$, Fig. 4B, b). This experiment shows directly that radial glial cells can undergo asymmetrical division to generate neurons that migrate along the radial glial fibre.

We also observed the morphology of dividing radial glial cells in time-lapse experiments. For example, we noted that radial glial fibres become extremely thin but do not seem to retract during mitosis. Although we cannot rule out the possibility that cells retracted and re-extended their radial processes in the interval between frames, a mechanism involving retention of radial fibres

may permit proliferative radial glia to maintain the specificity of their migrational guidance role during division.

The average clone size observed (4.77 ± 0.23 cells at 72 h after infection) can be explained by a series of asymmetric cell divisions, with radial glia dividing to generate one neuron per cell cycle (Fig. 4C, a). One cell cycle is required for retroviral gene expression, and only one daughter cell inherits the viral genome¹⁸. Assuming four cell cycles from E16 to E19 after viral incorporation¹⁹, one would therefore expect up to five cells per clone: four neurons and one progenitor. Some of the clones contained more than five cells (range 2 to 17, $n = 111$ clones), however, so other mechanisms may be involved. For example, symmetrical divisions of intermediate neural precursors may also occur (Fig. 4C, b). Although our data cannot exclude this possibility, our experiments using BrdU did not identify a separate population of non-radial glial proliferative cells in our clones. Therefore, the asymmetrical division model (Fig. 4C, a) seems to best explain our data.

Neuronal and glial lineages diverge early during neocortical development^{20,21}. Previous studies of neuronal and glial lineage relationships in neocortex did not examine radial glial lineages because either they were performed at stages when radial glial cells

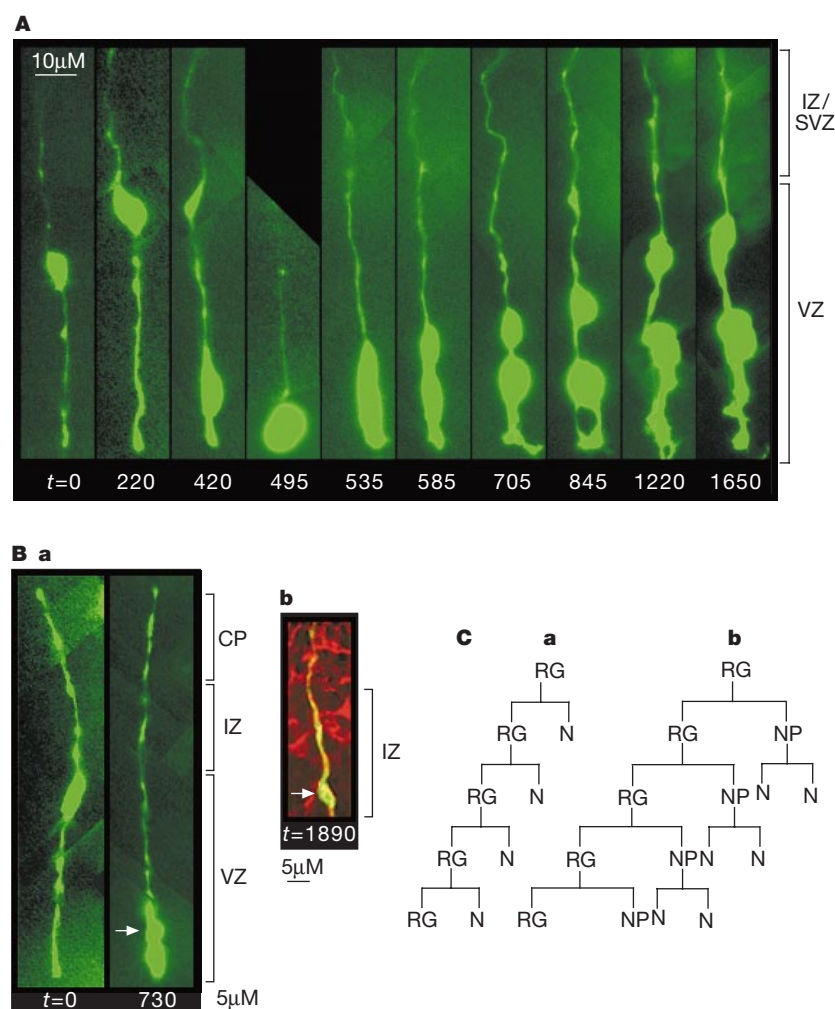


Figure 4 Time-lapse videomicroscopy of radial glial cell division. **A**, Single radial glial cell at 24 h after infection ($t = 0$). All radial glial cells imaged had an end-foot at the ventricular surface and a radial process extending to the pia. The radial glial cell descends to the ventricular surface, divides and translocates to the top of the VZ, while its daughter cell begins radial migration. Times are indicated (min). **B**, **a**, Another radial glial cell at 24 h after infection ($t = 0$), showing its fibre contact with both pial and ventricular surfaces.

b, After division, its daughter (arrows) migrated along the radial fibre into the intermediate zone and was confirmed to be TUJ1 positive. **C**, Two lineage tree diagrams illustrate potential division schemes by which a radial glial cell (RG) could produce successive generations of neurons (N), either directly (**a**) or through intermediate neural precursors (NP) (**b**). CP, cortical plate; IZ, intermediate zone.

are no longer present^{20,21} or they used markers incapable of identifying the fine processes of radial glia^{18,22}. Our data show that neuronal and radial glial lineages in the neocortex do not diverge early. Instead, similarly to observations in the adult avian brain^{9,23}, radial glial cells and newborn neurons in neocortex are clonally related. The generation of neurons by radial glia is supported by studies of the morphogen Notch, which establishes cell identity during asymmetric divisions between precursor cells and newborn neurons. Notch expression is believed to maintain cells in a proliferative state²⁴, and inhibition of Notch signalling causes early differentiation of neurons²⁵. Notch signalling in the developing cerebral cortex promotes radial glial cell identity²⁶, consistent with the concept that radial glial cells are neural precursors.

It has been widely assumed that radial glial cells and neuronal precursors in the developing neocortex represent distinct cell populations; however, radial glia have been suggested to be neuronal progenitors⁵, and radial glial cells have neural progenitor potential¹⁰. Our data provide direct evidence that radial glia are neuronal precursors *in vivo* at the peak of neurogenesis in the developing neocortex; they may even be the predominant neuronal precursors at this stage of development, but we cannot rule out the possibility that the retrovirus used here selectively infects a subpopulation of proliferating cells. The persistence of vimentin-positive radial glia in the adult hippocampus²⁷, a site where neurogenesis occurs throughout life, raises the possibility that the neurogenic potential of radial glia may extend into adulthood in some brain regions.

Previous studies of cell lineage in neocortex identified a subset of neurons that do not migrate along radial glial fibres, but instead migrate tangentially and disperse away from their site of origin in the VZ^{7,18}. Another group of tangentially migrating neurons in the developing neocortex is generated in the proliferative zone of the adjacent striatum²⁸. Thus, there appear to be many modes of cell migration in neocortex, both radial and tangential. Consistent with several routes for cortical migration, we found many dispersed GFP-labelled cells in addition to radial clones.

Radial glial cells and their associated migrating neurons constitute an invariant feature of developing mammalian cortex²⁹. The 'radial unit hypothesis'¹² predicts that clonally related neurons migrate along radial glial fibres to form functional cortical columns. Despite the discovery of additional routes for cortical migration^{7,18}, migration along radial glial fibres remains a central mechanism for establishing the architecture of the neocortex. Our findings establish that radial glial cells also generate neurons and act as migrational guides for their neuronal progeny. The dual function of this highly specialized cell may have wide-ranging implications for the study of neuronal migration disorders and therapeutic uses of progenitor cells. The clonal relationship between radial glial cells and neurons also helps confirm this grouping as a basic phylogenetic unit. The generation of neurons by radial glial cells provides a parsimonious mechanism by which local clonal relationships in the embryonic ventricular zone can be translated into functional columnar units in the adult neocortex.

Methods

Production of retrovirus

Replication-incompetent enhanced GFP-expressing retrovirus was produced from a stably transfected packaging cell line (293gp NIT-GFP; a kind gift from J. Goldman). Cells were transiently transfected at ~80% confluence with pVSV-G using the Calphos Mammalian Transfection kit (Clontech, Palo Alto, CA). Supernatant was collected 48 h after transfection, filtered through 0.45-µm low-protein binding filters (Fisher Scientific, Fair Lawn, NJ) and concentrated 100-fold at 50,000 g, 4 °C for 1.5 h. Pellets were resuspended in Opti-MEM (Gibco, Rockville, MD) and stored at -80 °C. Final titre of retrovirus was 5×10^5 to 1×10^6 colony-forming units per ml. Retroviral titres were adjusted to yield about 15 clones per mm².

Retroviral infection

Animals were maintained according to protocols approved by the Institutional Animal

Care and Use Committee at Columbia University. Uterine horns of E15- and E16-timed, pregnant Sprague Dawley rats (Taconic, NY) were exposed in a sterile biosafety level II hood. Retrovirus (0.5–1.0 µl) with Fast Green (2.5 mg ml⁻¹) (Sigma) was injected into the cerebral ventricles through a bevelled, calibrated glass micropipette (Drummond Scientific, Broomall, PA). After injection, the peritoneal cavity was lavaged with 10 ml of 0.9% NaCl, the uterine horns were replaced, and the wound was closed.

BrdU labelling

Embryos were injected with retrovirus on E16 as above, and 30, 36, 42 or 48 h after retroviral injections pregnant rats ($n = 9$) were given a single injection of BrdU (100 mg per kg, i.p.). Two hours later, embryos were removed and transcardially perfused with PBS pH 7.4, followed by 4% paraformaldehyde in PBS (PFA). Brains were fixed in PFA overnight and 100-µm sections were prepared on a Vibratome.

Immunohistochemistry

Embryos were removed 1–4 d after infection, perfused and sectioned as above. Sections were blocked in 10% serum, 0.1% Triton-X and 0.2% gelatin, and incubated for 48 h in the following antibodies: anti-TUJ1 1:40 (Babco, Richmond, CA); anti-nestin 1:40 (Becton Dickinson, Franklin Lakes, NJ); anti-vimentin 1:40 (Sigma); or anti-GFAP 1:40 (Sigma). Sections were washed and incubated in Texas-red-conjugated anti-mouse or anti-rabbit secondary antibodies (Vector, Burlingame, CA). Sections for BrdU staining were treated as described¹⁶. Sections were mounted on glass slides with coverslips (Aqua-mount; Lerner Labs, Pittsburgh, PA) before imaging.

Confocal microscopy

Sections were imaged on a Zeiss 410 laser-scanning confocal microscope. Excitation/emission wavelengths were 488/515 nm (GFP) and 568/590 nm (Texas red and Alexa). Z-series images were collected at 1-µm steps on a PC using Zeiss LSM software. To show co-labelling by GFP and cellular markers, only Z sections in the same focal plane as the GFP-labelled cell bodies were used for producing figures. Images were contrast enhanced and assembled into montages, and false colour was applied using Photoshop 5.0 software (Adobe Systems).

Electrophysiology

Whole-cell recordings from living brain slices were obtained as described³⁰. Briefly, embryos were removed 2–4 d after injection, brains were dissected, and slices were cut coronally at 250–300 µm on a Vibratome. Alexa 594 or Neurobiotin (Molecular Probes, Eugene, OR) were added to electrode solutions to identify recorded cells. GFP-expressing cells were visualized by epifluorescence on an upright, fixed-stage microscope and selected for IR-DIC mediated recording. Neurobiotin was visualized by processing with NeutraLite avidin (Molecular Probes).

Time-lapse imaging of GFP-expressing clones

Embryos were injected with retrovirus on E16, and coronal slices prepared 24 h later as above. Slices were placed on Millicell-CM inserts (Millipore, Bedford, MA) in culture medium containing 25% Hanks balanced salt solution, 47% Basal modified Eagle's medium, 25% normal horse serum, and 1× Pen/Strep/Glutamine (Gibco), 0.66% glucose, and 10 ng ml⁻¹ bFGF (Collaborative Biomedical, Bedford, MA). Images of radial units were captured on an epifluorescence microscope using Scion Image software at intervals of 20–120 min for 60 h. Epifluorescent images from several focal planes were assembled in montages in Photoshop 5.0 to produce sharp images. Slices were incubated at 37 °C in 5% CO₂ between imaging. Slices were fixed, immunostained and imaged on a confocal microscope as above.

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The gating mechanism of the large mechanosensitive channel MscL

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The mechanosensitive channel of large conductance, MscL, is a ubiquitous membrane-embedded valve involved in turgor regulation in bacteria^{1–5}. The crystal structure of MscL from *Mycobacterium tuberculosis*⁶ provides a starting point for analysing molecular mechanisms of tension-dependent channel gating. Here we develop structural models in which a cytoplasmic gate is formed by a bundle of five amino-terminal helices (S1), previously unresolved in the crystal structure. When membrane tension is applied, the transmembrane barrel expands and pulls the gate apart through the S1–M1 linker. We tested these models by substituting cysteines for residues predicted to be near each other only in either the closed or open conformation. Our results

demonstrate that S1 segments form the bundle when the channel is closed, and crosslinking between S1 segments prevents opening. S1 segments interact with M2 when the channel is open, and crosslinking of S1 to M2 impedes channel closing. Gating is affected by the length of the S1–M1 linker in a manner consistent with the model, revealing critical spatial relationships between the domains that transmit force from the lipid bilayer to the channel gate.

A simplified ‘mechanistic’ model of a MscL gating process is illustrated next to a typical single-MscL current event in Fig. 1. The central gate is composed of five identical sections, one per subunit. The gate’s sections are connected to the channel’s transmembrane rim by radial strings. Membrane tension first stretches the rim from the resting closed conformation (C) to a closed–expanded (CE) conformation. The first substate (S1) can occur when one section of the gate breaks away. The rest of the gate’s assembly can then disrupt quickly, leading to the fully open state (O) that displays short, intermittent subconductances when individual sections of the gate partly occlude the pore. This scheme is supported by several findings. Thermodynamic analysis of tension sensitivity of two major rate constants of the MscL channel of *Escherichia coli* (EcoMscL) opening predicts that the channel expands to at least two-thirds of its open size before opening⁷. The channel often passes through substates before opening completely and flickers to substates once opened. The protein expansion area ($\sim 6 \text{ nm}^2$) calculated for the electrically detectable opening transition, CE→O in this scheme, is smaller than the cross-sectional area of the open pore ($\sim 7\text{--}12 \text{ nm}^2$ or a pore diameter of 3–4 nm) approximated from the conductance of the open state⁸ and permeability of the pore to large molecules⁹. Time-resolved recordings reveal that the transitions between the low subconducting (S1) and fully open (O) states are relatively independent of tension, indicating that the major increase in conductance does not involve substantial changes in the outer dimension of the channel complex⁷.

Features of this gating hypothesis were incorporated into a series

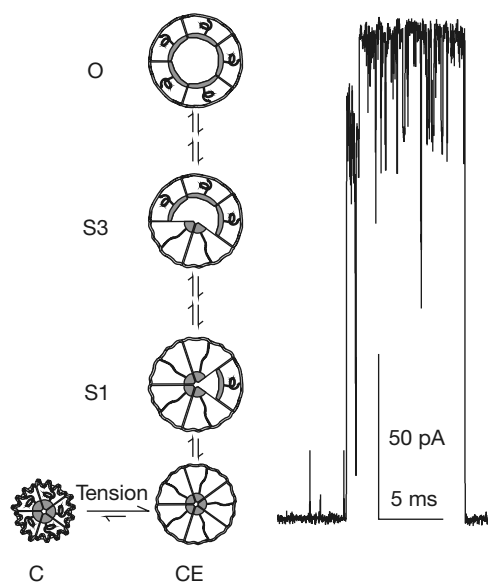


Figure 1 MscL gating represented ‘mechanistically’. The channel has five identical subunits, each contributing to the closed gate assembly in the centre and the elastic outer rim on the periphery. The transmembrane rim must expand substantially before stress in linkers that connect the rim to the gate pulls the gate assembly apart. The channel reaches the open conformation (O) through a series of short-lived subconducting states (S1, S2, etc.) as the central gate breaks apart. A typical single MscL current (right) recorded at -50 mV shows an opening through a substate followed by flickering to substates once the channel has opened.

of molecular models. The transmembrane region of the modelled closed resting conformation of EcoMscL closely resembles the crystal structure of the pentameric TbMscL homologue (Fig. 2a). Models of transition and open conformations of Fig. 2 were developed by using the secondary structure of the closed conformation and maintaining most of the interactions between the tightly packed M1 and M2 segments of adjacent subunits. Our principal conclusion was that the channel gate is unlikely to be formed solely by the inner part of the M1 segment, as proposed previously^{5,9}. The ability to expand 'silently' and then open without substantially changing area implies the presence of a second gate. The highly conserved S1 segment is the most likely candidate for an additional gate. The periplasmic S2 segment is poorly conserved and does not occlude the pore in the crystal structure, whereas another cytoplasmic segment, S3, is dispensable¹⁰. Thus, we modelled the S1 segments as a bundle of short amphipathic α -helices that occludes the pore when the channel is closed.

The opening of a large pore, which requires the separation of central M1 helices, was modelled as a gradual increase in helical tilts associated with an iris-like movement of M1 helices away from the central axis of the pore (Fig. 2). The '4-4 ridges-into-grooves' packing¹¹ between adjacent M1 helices, maintained throughout the transition, allows a gradual expansion of the barrel by the gap-free sliding of transmembrane helices one along another. Alternative models in which all ten transmembrane helices line the pore throughout the transmembrane region did not satisfy our modelling criteria¹². To compensate for the shortening of the barrel, we postulate that the periplasmic and cytoplasmic segments move into the transmembrane region, where hydrophobic surfaces of S2 and S3 interact with lipids. When tension deforms the barrel, the S1 to M1 linkers (residues Arg 13-Gly 14-Asn 15, corresponding to strings in Fig. 1) become maximally extended. The S1 bundle can then switch from its most compact left-handed conformation (Fig. 3a) to a wider right-handed conformation (Fig. 3b). As the gate bundle disrupts, S1 segments swing away from their pore-blocking axial position and dock on the barrel wall. Descriptions and methods used to develop these models will be presented elsewhere¹² (for details and atomic coordinates see Supplementary Information).

The hypothesis that the S1 bundle occludes the pore was tested by

substituting cysteines for specific S1 residues and determining whether these residues are proximal in adjacent subunits in closed channels, and if so, whether crosslinking of S1 segments prevents channel opening. Individual residues in the core of the bundle with β -carbons less than 7 Å apart should crosslink subunits when replaced with cysteine. The left-handed bundle (Fig. 3a) is supported for the resting conformation by our finding that both F7C and F10C mutants formed disulphide bridges spontaneously, as revealed on western blots after protein separation by non-reducing

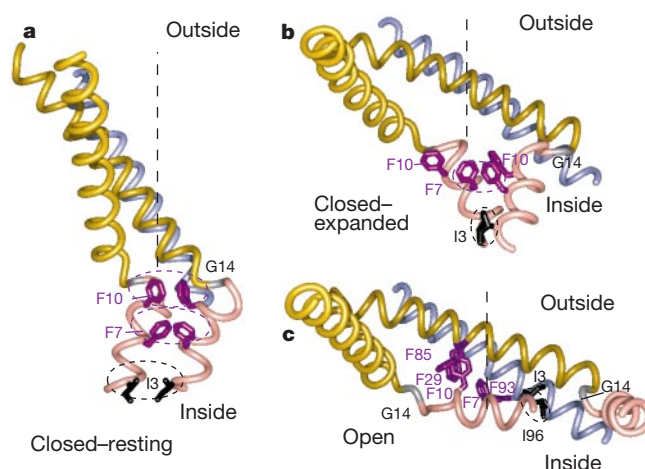


Figure 3 Residue interactions in three predicted conformations. S1 (pink) and M1 (yellow) segments of two adjacent subunits and M2 (light blue) from a third subunit are shown as a side view from the inside of the pore towards the channel wall. The Gly 14 residue in the centre of the S1-M1 linker is grey. The dashed line indicates the axis of five-fold symmetry. Ile 3 (black), Phe 7 and Phe 10 residues (purple) interact in closed-resting (a) and closed-expanded (b) conformations. c, The open conformation is stabilized by the cluster of phenylalanines and interaction between Ile 3 of S1 and Ile 96 (black) of M2. Pairwise interactions supported by cysteine crosslinking are circled. The β -carbons of all of these pairs are less than 7 Å apart. Amino acids are shown with single-letter abbreviations.

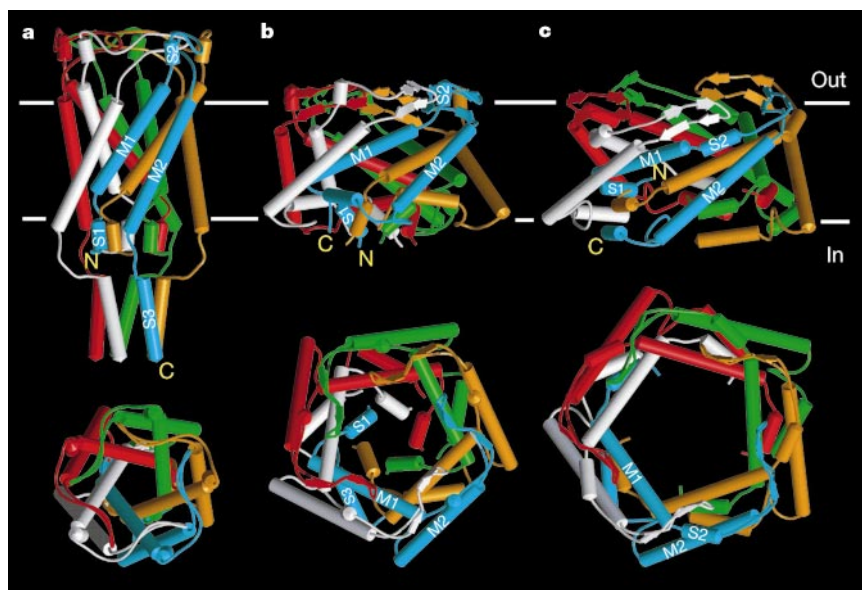


Figure 2 Molecular models of EcoMscL backbone. a, Closed-resting conformation; b, closed-expanded conformation; c, open conformation. The views from the side (upper row) and from the periplasm (bottom row) show all five subunits coloured individually. Helical segments S1, M1, S2, M2 and S3 are cylinders on the subunit nearest to the

viewer (blue) with the positions of the amino (N) and carboxy (C) termini (upper row only). Horizontal lines show approximate positions of the inner and outer boundaries of the membrane. The modelled path for the transition illustrates a substantial in-plane expansion of the complex before movement of the S1 gate unblocks the pore.

SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 4a). In spite of abundant expression, no MscL currents could be evoked in F7C and F10C spheroplasts until the patch was incubated with a reducing agent. Figure 4b shows that F10C disulphide bridges were reduced slowly with 200 mM 2-mercaptoethanol in the pipette. Partial openings appeared gradually, presumably owing to the reduction of one of two possible disulphide bridges (12 of 17 patches, 30 min incubation). These were followed in about 1 h by complete 3.5 nS openings (three of three patches). The reaction was even slower with mercaptoethanol in the bath instead of the pipette, showing that the F10C residues, although located on the cytoplasmic side, are more accessible through the pore from the periplasm. The observation that the channel opens incompletely when only two S1 segments were crosslinked (after partial reduction) supports the hypothesis that subconductance states occur when some, but not all, of the S1 segments partly occlude the pore. The F7C mutant was fully activated within 30 min with 200 mM mercaptoethanol in the pipette, and the reaction was faster when a moderate suction of ~50 mm Hg (~6,700 Pa) was applied to the pipette (five of eight patches; data not shown). This indicates that the F7C structure has more conformational freedom than that of F10C, being able to expand to a larger pore (see Fig. 3b). S2C and I3C mutants also led to the formation of subunit dimers in the presence of the oxidizing

agent iodine (I_2) and double cysteine mutants such as I3C/I4C or E6C/F7C readily formed subunit trimers and tetramers (Fig. 4a), indicating that several S1 segments are positioned next to each other. In the control, the I3C mutant showed moderate coupling on westerns blots. With no treatment or in the presence of mercaptoethanol in the bath (100 mM) we observed complete MscL openings (three of four patches; Fig. 4c, upper trace). After exposure of the patch to I_2 (1 mM, 30 min) in the bath we observed only no openings or incomplete ones (four of four patches; bottom trace). Iodine or mercaptoethanol had no detectable effects on wild-type MscL, although both agents decreased patch stability.

The model predicts that S1 segments dock next to M1 and M2 and form part of the channel's lining when it opens. Specific interactions in open channels between S1, M1 and M2 were predicted primarily on the basis of energetically favourable interactions between highly conserved phenylalanine residues (Phe 7, Phe 10, Phe 29, Phe 85 and Phe 93; see Fig. 3c). These interactions cannot be examined by cysteine substitutions because F7C and F10C mutants spontaneously lock in a closed conformation. We identified another hydrophobic residue, Ile 3 (on S1), that the open conformation model predicts should interact with Ile 96 (on M2 of a different subunit). We generated an I3C/I96C double mutant and studied possible coupling between cysteines, both with a patch-

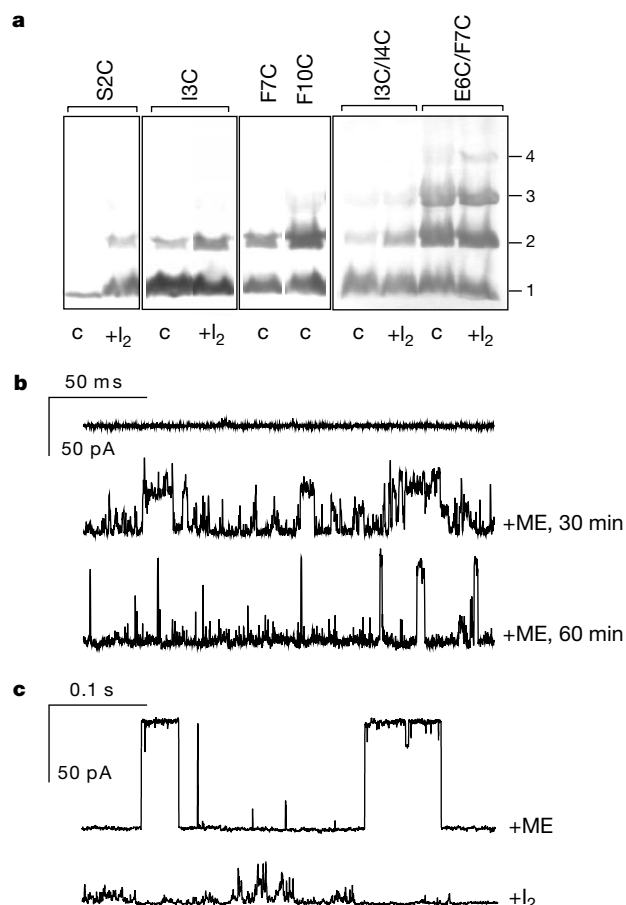


Figure 4 Disulphide trapping of S1 segments of MscL. **a**, Western blot patterns of intersubunit crosslinking in single and double cysteine mutants. Numbers at the right denote the degree of subunit multimerization. Control samples (c) received no treatment; other samples (+ I_2) were treated with 0.3 mM I_2 after French pressing. **b**, Traces obtained with F10C channels after incubation for 0 min (top), 30 min (middle) and 60 min (bottom) with 200 mM mercaptoethanol (ME) in the pipette. The pipette pressures were between 300 and 340 mm Hg (~40 and ~45 kPa). **c**, I3C channel activities recorded in the control patch (upper trace) and in the presence of 1 mM I_2 in the bath after incubation for 30 min (lower trace).

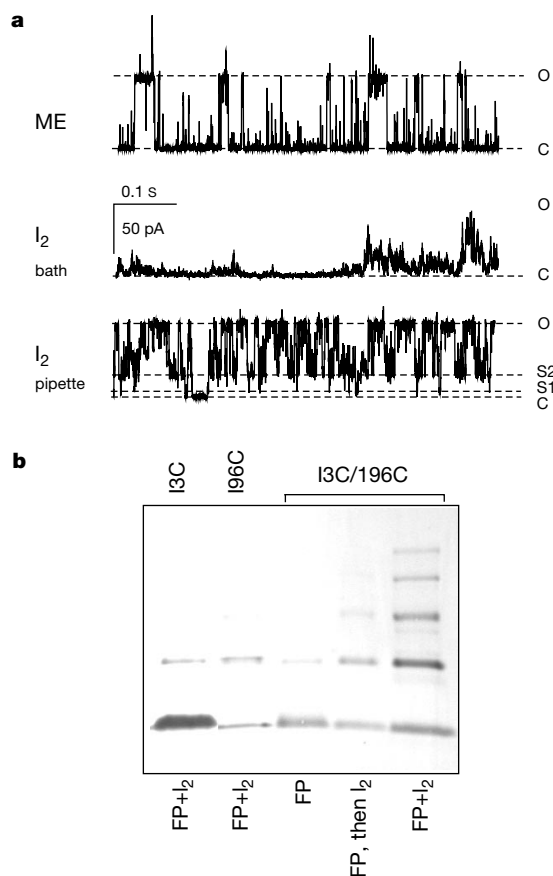


Figure 5 S1 interacts with M2 in the open state. **a**, Patch-clamp examination of I3C/I96C spheroplasts. In the presence of 0.1 M ME, in both the bath and the pipette (upper trace), MscL openings are of normal amplitude. The middle trace corresponds to the patch exposure to I_2 (1 mM, 30 min) in the bath and displays only incomplete openings. The bottom trace was obtained with 0.2 mM I_2 in the pipette ($p = 400$ mm Hg (~53 kPa)) and shows the channel 'locked' at the second substate. A release of pressure still results in a complete closure of the channel. **b**, Western blot after crosslinking of I3C and I96C mutants and the I3C/I96C double mutant. Expresser cells were French-pressed with (FP + I_2), without (FP), or before (FP, then I_2) the addition of 0.5 mM I_2 .

clamp and biochemically (Fig. 5). When disulphide bridging was prevented by 100 mM mercaptoethanol, both in the pipette and in the bath, the gating pattern of the double-mutant channel was nearly normal. With I_2 in the bath (0.1–0.3 mM), we observed mostly partial openings, indicating that complete opening was inhibited by the formation of disulphide bridges at position 3 on different subunits, as predicted for the closed conformation (Figs 3a, 4a, c). The addition of I_2 to the pipette rather than to the bath changed the gating pattern qualitatively. The bottom trace of Fig. 5a illustrates that the channel gated predominantly between the fully open state and the second substate and rarely closed completely (observed in four of nine patches with sustained channel activity for more than 1 min). This type of behaviour was never observed in any of the control patches with wild-type MscL. I_2 in the pipette had no detectable effect on the currents of single I3C (nine patches) or I96C (six patches) mutants. These results support our model because I_2 in the pipette should readily attack I3C–I96C pairs either through the lipid or the pore when the channel is open but should not reach I3C when the channel is closed.

In the biochemical approach, trapping of MscL subunits in expanded or open conformations was achieved by passing the expresser cells through a French press in the presence of I_2 . The mechanical stress that causes cell disruption is assumed to open MscL transiently. Single I3C and I96C mutants showed only a moderate quantity of dimers formed after French pressing in the presence of I_2 (Fig. 5b). A substantial multimerization (up to

fivefold) of double-mutant subunits was obtained under the same conditions, as expected if the disulphide bridge between residues 3 and 96 formed between different subunits (see Fig. 3c).

Tension through the S1–M1 linker (residues Arg 13–Gly 14–Asn 15) is predicted to pull the S1 gate apart once the transmembrane region has expanded substantially. If so, then gating of the channel should depend on the length of the linker, with longer linkers making it less sensitive to stretch or favouring subconductances (see mechanical model in Fig. 1). It has been shown that a MscL mutant with a shorter linker (with Gly 14 deleted) often enters a regime in which it does not close completely¹³. We extended the linker by the insertion of additional glycine residue (the GG14 mutation), and found that, when the membrane was stretched, the mutant had a much higher propensity for low-conducting and intermediate substates as illustrated by the traces and occupancy histogram in Fig. 6. Apparently, the longer linkers allow more freedom for S1 segments to recombine in transient semi-closed conformations as illustrated in Fig. 1. Further extension of the linker by the insertion of an AG pair next to Gly 14 (GAG14 mutation) greatly impeded the opening of the channel. GAG14 channels opened only transiently and then inactivated (data not shown).

Experiments presented here indicate that S1 segments interact with each other in the closed conformation and that the S1 bundle disrupts when MscL opens fully. The subconductances probably represent the dynamics of reassociation of individual S1 segments. The model implies that the ‘elastic’ barrel composed of tilted transmembrane helices acts as a tension sensor. Force is conveyed to the S1 gate only when expansion of the barrel fully extends the S1–M1 linkers. This might explain the steep, almost threshold-like dependence of channel activity on tension⁷. The length of the linker is crucial to MscL’s finely tuned gate and is invariable in all 35 known MscL homologues. The entire cluster of residues encompassing S1, linker and the cytoplasmic half of M1 is the most conserved part of the channel^{5,12,14}.

The TbMscL crystal structure indicates that a constriction in the cytoplasmic half of the M1 pore poses a major hydrophobic barrier to ion permeation^{5,6}. The hypothesis that this region forms an ‘M1 gate’ has been supported by mutagenesis experiments in which disruption of the hydrophobic ‘lock’ (formed primarily by Val 23 in EcoMscL) by hydrophilic or charged substitutions causes the channel to activate readily to subconducting states^{15,16}. However, mutants with a disrupted M1 gate still gate and close completely, indicating that the second, S1, gate remains functional. Why does the channel need two gates? MscL serves as ‘safety valve’ in bacterial cells² and combines two extreme features. When open at high sublytic tension, it passes high current and organic osmolytes to release excess pressure quickly. However, it resides in the energized cytoplasmic membrane, and therefore at rest it must be perfectly shut to maintain electrical integrity of the membrane. The presence of two gates in series might be required to make MscL absolutely leak-proof in a wide range of subthreshold tensions and therefore safe for bacteria. □

Methods

Modelling

Initial models were developed with PSSHOW and were minimized with Charmm¹⁷ (for details and atomic coordinates for the models see Supplementary Information). Molecular graphics images of Fig. 3 were produced with the MidasPlus program (Computer Graphics Laboratory, University of California, San Francisco, California, USA (supported by NIH)).

Genetic manipulations

Native MscL has no endogenous cysteine residues. All genetic modifications of *mscL* were done in the pB10b vector, in which the open reading frame was cloned as a *Bgl*II–*Xho*I fragment downstream of the inducible P_{lacUV5} promoter¹⁵. Single and double cysteine substitutions were constructed with the PCR-based ‘megaprimer’ mutagenesis technique as described in ref. 16 and verified by sequencing. All mutants were expressed in *E. coli* PB104 *mscL*[−], *recA*[−] cells¹⁸.

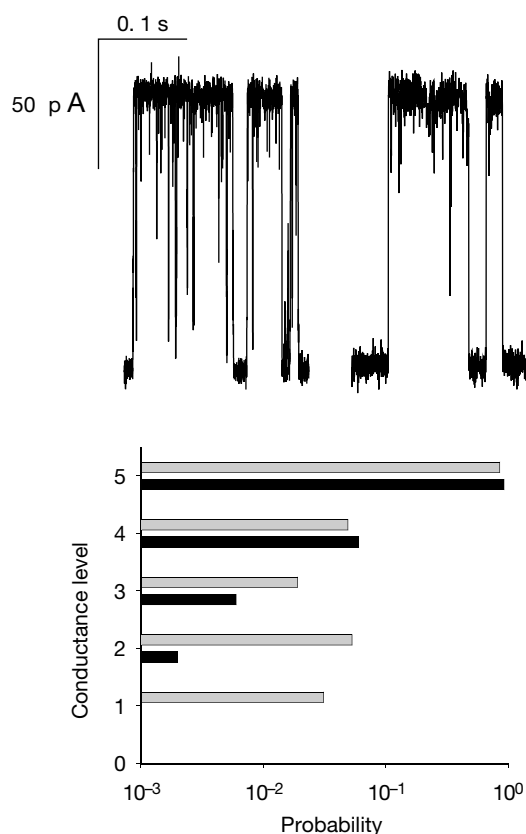


Figure 6 The GG14 mutant with extended S1–M1 linker shows higher occupancies of lower sub-conducting states. Single-channel traces were recorded at -20 mV in a symmetrical buffer containing 0.4 M KCl (see Methods), in a bandwidth of 20 kHz at $P_0 \sim 0.1$. The occupancy of five major conducting states was analysed over ~ 100 opening events with the SKM protocol, part of the QuB kinetic analysis suite. The conducting levels 0–6 on the plot correspond to the C, S1, S2, S3, S4 and O conducting states of MscL (see Methods). The GG14 mutant (grey bars) visited the three lower substates substantially more often than did wild-type MscL (black bars).

Disulphide trapping

Disulphide crosslinking experiments were performed with *E. coli* membranes with iodine (I_2) as an oxidizing agent¹⁹. Isopropylthiogalactoside-induced expresser cells ($D_{600} \sim 0.5$, about 8 ml per sample) were washed, resuspended in 1 ml of crosslinking buffer (25 mM KH_2PO_4 , 25 mM K_2HPO_4 , 5 mM $MgCl_2$ (pH 7.2)) and subjected to rupture in a French press. To crosslink MscL in expanded and/or open states, the cells were stressed and ruptured by passing them through a narrow slit in a French press. I_2 (0.5 mM) was added a few seconds before the procedure. To trap the subunits in the resting closed conformation, membranes were incubated in I_2 at 20 °C for 15 min beginning at 15 min after French pressing. The membranes were pelleted and dissolved in 200 μ l of non-reducing SDS sample buffer containing 0.2 M sodium iodoacetate and 4 M urea. Samples were heated to 70 °C for 10 min and resolved by SDS–PAGE. The products of subunit crosslinking were detected by western blots by using a Promega AP kit with antibodies directed to the carboxy terminus of MscL²⁰.

Single-channel recording and analysis

Giant *E. coli* spheroplasts were prepared and examined with patch-clamp as described²¹. Single-channel traces were recorded with a bandwidth of 20 kHz at -20 mV (or at -50 mV, as in Fig. 1) in symmetrical (the same on both sides of the membrane) 0.2 M KCl, 100 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM HEPES (pH 6.0). All measurements were made at room temperature. Many of the cysteine mutants studied here activated at slightly higher pipette pressures, approaching the limit for patch stability. In the statistics presented in the text, only patches that sustained 280–300 mm Hg (~ 37 –40 kPa) for at least 30 s were counted as successful attempts. Currents were recorded with Pclamp 6 software. For the substate analysis (Fig. 6), recordings were made in the same buffer containing 400 mM KCl. The traces were idealized and the occupancy of major conducting states was analysed over ~ 100 opening events with the SKM protocol, part of the QuB kinetic analysis suite (developed by F. Qin, A. Auerbach and F. Sachs at SUNY-Buffalo). A combined analysis of wild-type, GG14 and I3C/196C MscL channel records showed that MscL can potentially occupy seven conducting states with approximate conductances of 0 (C), 0.09 (S1), 0.25 (S2), 0.56 (S3), 0.74 (S4), 0.89 (S5) and 1.0 (O), with the fully open state taken as unity. In the previous analysis⁷ S1 and S3 were missing owing to very low occupancies of these substates in the wild type.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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macho-1 encodes a localized mRNA in ascidian eggs that specifies muscle fate during embryogenesis

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Maternal information stored in particular regions of the egg cytoplasm has an important function in the determination of developmental fate during early animal development^{1,2}. Ascidians show mosaic development^{3,4}; such autonomous development has been taken as evidence that prelocalized ooplasmic factors specify tissue precursor cells during embryogenesis. Interest has been concentrated on the mechanisms underlying the formation of muscle cells in the tail, as yellow-coloured myoplasm in eggs is preferentially segregated into muscle-lineage blastomeres⁵. Here we show that maternal messenger RNA of the macho-1 gene is a determinant of muscle fate in the ascidian *Halocynthia roretzi*. The macho-1 mRNA encodes a zinc-finger protein, and the mRNA is localized to the myoplasm of eggs. Depletion of the mRNA specifically resulted in the loss of primary muscle cells in the tail, as shown by the expression of muscle-specific molecular markers. The myoplasm of macho-1-deficient eggs lost its ability to promote muscle formation. Injection of synthesized macho-1 mRNA caused ectopic muscle formation in non-muscle-lineage cells. Our results indicate that macho-1 may be both required and sufficient for specification of muscle fate, and that the mRNA is a genuine, localized muscle determinant.

The embryos of ascidians develop into tadpole larvae, which display a primitive chordate body plan⁶. In this group of animals, developmental fate is coupled to the lineal descent of a cell, and specification of fate is achieved cell autonomously. This is supported by experiments where blastomeres acquire invariant fates, even in isolation from the embryo. Autonomous specification suggests that the developmental fates of blastomeres are determined by the inheritance of factors that are prelocalized in the egg cytoplasm and apportioned to different cells during cleavage. Direct evidence for the presence of maternal cytoplasmic determinants has been obtained by cytoplasmic transfer. Figure 1c–e summarizes the results of systematic analysis of the distribution of determinants for muscle that have been shown by cytoplasmic transfer experiments⁷. Briefly, the results show that muscle determinants are already present and localized in the uncleaved egg. Just after fertilization, these determinants are concentrated at the vegetal pole, then move to the future posterior pole during ooplasmic segregation, and finally settle at sites that correspond to the appropriate region in the future fate map. Muscle determinants are partitioned into muscle progenitor blastomeres during subsequent cleavages.

To isolate localized maternal messages concentrated at the vegetal

hemisphere, we carried out subtraction hybridization screening. Our report analyses one of the clones that we obtained, named macho-1. The macho-1 protein has five CCHH-type zinc-finger repeats (Fig. 1a, b). A BLAST search indicated that the zinc-finger domain of macho-1 shows highest similarity (70% identical, 80% similar) to that of vertebrate *Zic* family proteins⁸, which are transcription factors expressed mainly in the nervous system. The sequence of macho-1 outside the zinc-finger domain showed no homology to *Zic* proteins or to other known proteins. All *Zic* family proteins have another conserved domain outside the zinc-finger domain, but this is not present in macho-1. It is therefore unlikely that macho-1 is an orthologue of vertebrate *Zic*. In *H. roretzi*, another gene (*HrZicN*) whose zinc finger is slightly more similar to vertebrate *Zic* has been isolated (S. Wada and H. Saiga, personal communication). The expression of the gene is temporally and spatially comparable to that of vertebrate *Zic*. Therefore, macho-1, *HrZicN* and vertebrate *Zic* may have originated from the same ancestral gene, but the function of macho-1 is greatly diverged (see below).

The distribution of maternal macho-1 mRNA in eggs is shown in Fig. 1f–h, and corresponds closely to the distribution of muscle determinant (Fig. 1c–e). Loss-of-function-type experiments offer a

straightforward approach examining the function of genes. We have devised a method for depleting maternal mRNAs in ascidians by injection of antisense oligodeoxynucleotides. Unfertilized eggs were injected, kept overnight and fertilized the next day after a time sufficient for the depletion of the targeted RNA. We used scrambled and sense oligodeoxynucleotides as controls.

The macho-1 antisense oligonucleotide was effective in removing the target mRNA from eggs and embryos, as shown by *in situ* hybridization with the macho-1 probe (Fig. 2a–c). We then confirmed the specificity of the depletion. First, sense and scrambled oligonucleotides did not deplete macho-1 mRNA in the eggs (Fig. 2d, e). Second, in eggs injected with antisense oligonucleotide and then probed for *HrWnt5* and 001D19 mRNAs, which showed a similar localization pattern to macho-1 transcripts, were completely unaffected (Fig. 2f, g). This also confirmed that the ooplasmic segregation and localization machinery of the embryos had not been altered by oligonucleotide injection.

Ooplasmic segregation and cleavage pattern were normal in macho-1-depleted eggs. The eggs underwent gastrulation, and embryogenesis seemed normal up to the neurula stage. However, in tailbud embryos, tail formation was severely affected. *Halocynthia* tadpole larvae, consisting of about 3,000 cells, hatch 35 h after

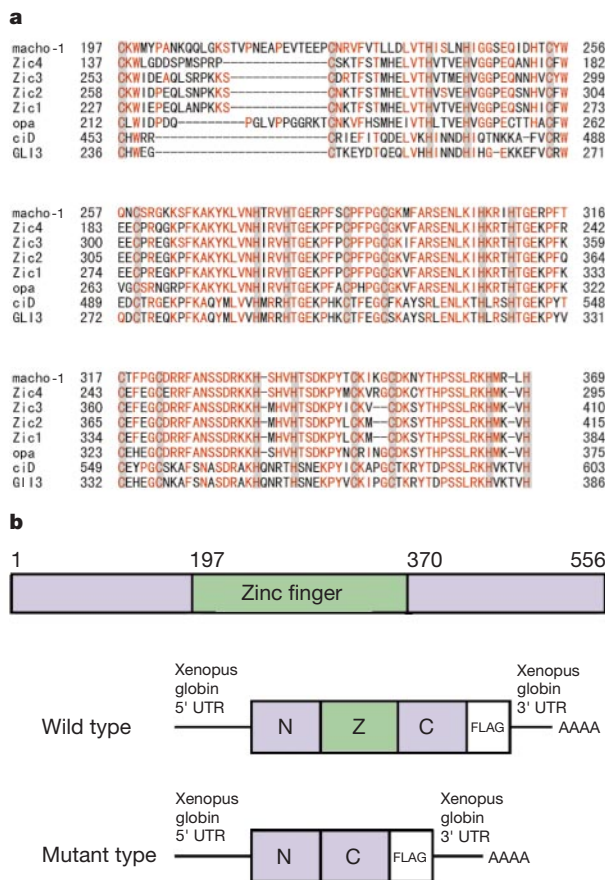
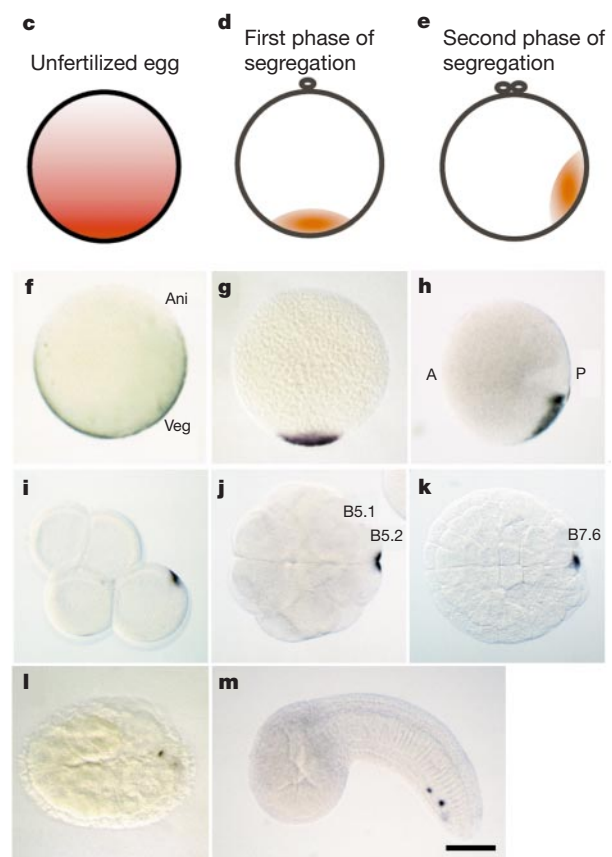


Figure 1 Structure and localization of macho-1 transcript. **a**, Alignment of zinc-finger domain of predicted amino-acid sequence of macho-1, mouse *Zic4*, *Zic3*, *Zic2*, *Zic1*, *Drosophila odd-paired* (*opa*), *cubitus interruptus* (*ciD*) and chick *Gli3* (accession numbers Q61467, Q62521, Q62520, P46684, P39768, P19538 and P55879, respectively). Identical residues between macho-1 and others are indicated (red). The cysteine and histidine relevant to the zinc-finger domain are shaded. **b**, Structure of macho-1 protein and constructs for *in vitro* mRNA synthesis. Macho-1 has five zinc fingers in the central part. The cDNA clone is 2,210 bp long and encodes a 556-amino-acid ORF. The wild-type construct encodes the full length, but the mutant construct lacks the zinc-finger domain. **c–e**, Localization of muscle determinants in unfertilized egg (**c**), and eggs after the first (**d**)



and second (**e**) phase of ooplasmic segregation⁷. Animal pole is top, and posterior is to the right. **f–h**, Distribution of maternal macho-1 mRNA shown by *in situ* hybridization in eggs at stages corresponding to **c–e**. A, anterior; P, posterior. **i–m**, Localization of macho-1 mRNA during embryogenesis. Anterior is to the left. **i**, Eight-cell stage, lateral view. *In situ* hybridization signal is localized to tiny spots in B4.1 blastomeres. Sixteen-cell stage (**j**) and 110-cell stage (**k**) embryos shown in vegetal view. Staining is restricted to the B7.6 blastomeres that do not divide further during embryogenesis. **l, m**, Late gastrula and early tailbud stage. mRNA is observed in two cells in the endodermal strand derived from the B7.6 blastomeres in **k**. No zygotic expression of macho-1 is observed.

fertilization. Each tadpole has 42 muscle cells in the tail that express acetylcholinesterase (AChE)⁹, actin¹⁰ and myosin¹¹ (Fig. 3a, b). At hatching, the trunk region seemed normal but the tail was shortened in macho-1-depleted larvae (Fig. 3c). Formation of various tissues, epidermis, sensory pigment cells, notochord and endoderm was similar to that in normal larvae. The larvae were surrounded by epidermis. Formation of sensory pigment cells and the notochord

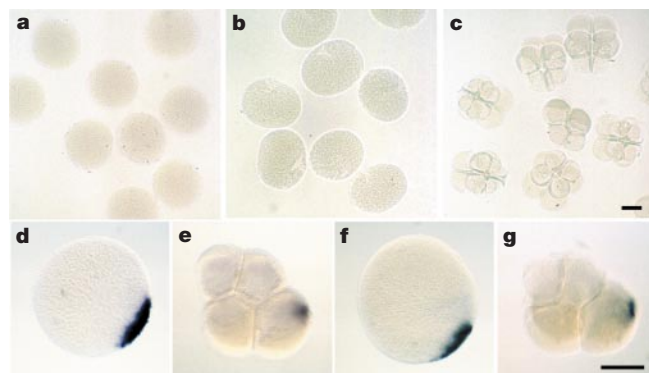


Figure 2 Depletion of maternal mRNA by antisense oligonucleotide. **a–c**, Antisense oligonucleotide-injected unfertilized eggs (**a**), eggs after the second phase of ooplasmic segregation (**b**) and eight-cell embryos (**c**) probed for macho-1 mRNA. **d, e**, Sense oligonucleotide-injected controls at the second phase (**d**) and the eight-cell stage (**e**). **f, g**, Antisense oligonucleotide complementary to macho-1 was injected, then the specimens were probed for *HrWnt-5*, whose mRNA shows a similar localization pattern as macho-1 mRNA. Scale bars, 100 μm.

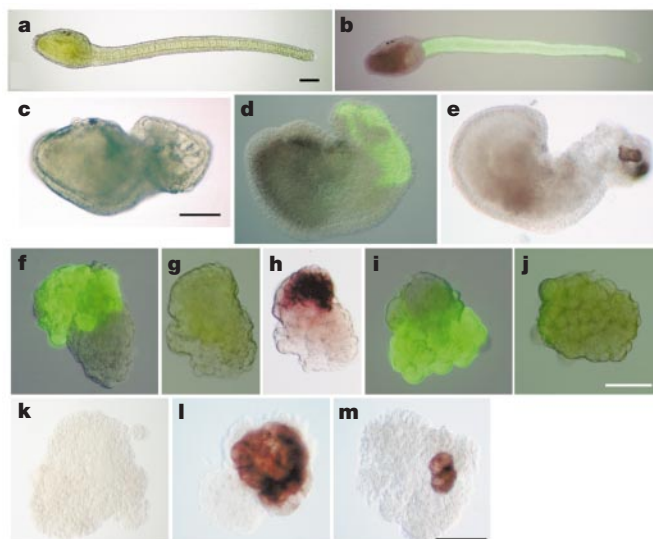
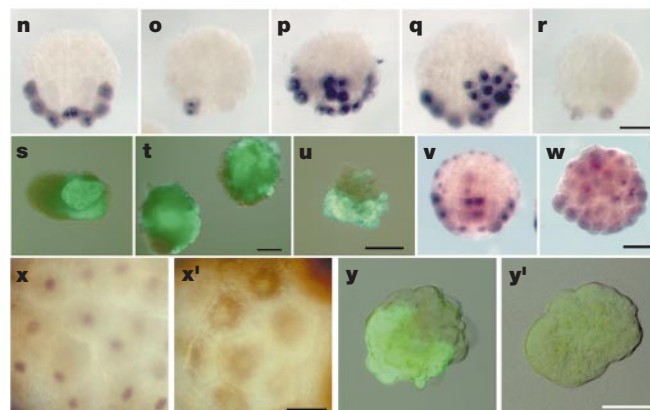


Figure 3 Depletion of macho-1 mRNA results in loss of primary muscle cells, and its overexpression causes ectopic muscle formation. **a**, Developed tadpole larva from egg injected with scrambled oligonucleotide. **b**, Larva injected with scrambled oligonucleotide and stained with anti-myosin antibody (green) and for endoderm-specific alkaline phosphatase (ALP) activity (brown). **c–e**, Larvae derived from antisense oligonucleotide-injected eggs. **c**, Sensory pigment cell and notochord are visible. **d**, Larva with ALP-positive endoderm and reduced amount of myosin-positive cells. **e**, Larva with AChE-positive muscle cells at the tail tip. **f–j**, Partial embryos developed from isolated B4.1 blastomeres of eight-cell embryos. Specimens were stained with anti-myosin antibody except for **h**. Scrambled oligonucleotide (**f**) and antisense oligonucleotide (**g**) were injected. **h**, Same partial embryo as in **g**, stained for ALP. **i**, Deficiency of muscle formation was compensated for by subsequent injection of synthesized macho-1 mRNA. **j**, Muscle formation is not rescued by injection of mutant mRNA lacking zinc-finger domain. **k–m**, B4.1 partial embryos corresponding to **g**, **i** and **j**, respectively, stained for AChE.

was morphologically evident. Specimens that were histochemically stained for alkaline phosphatase¹² confirmed that endoderm formation was normal (Fig. 3b, d). To determine the events that had occurred in the shortened tail region, macho-1-deficient larvae were stained with anti-myosin antibody. The amount of muscle was significantly reduced in over 70% of the embryos (Fig. 3d). Reduction of muscle was further confirmed by histochemical staining for AChE, a muscle-specific enzyme (Fig. 3e).

Although muscle was reduced, some muscle cells were always present and were often located at the tip of the tail. There are two types of muscle cells in the larval tail: primary and secondary muscle cells¹³. The primary muscle cells (28 out of the total 42 muscle cells) are derived from B-line (posterior–vegetal) blastomeres of the eight-cell embryo (see Fig. 5 below). Formation of the primary muscle shows cell autonomy, and the fate is specified by localized muscle determinants. However, the 14 secondary muscle cells located at the tip of the tail are derived from b- and A-line blastomeres, and their fate is determined by cell interactions, probably during gastrulation¹⁴. Thus, it is plausible that the muscle cells that form at the tip of the tail in macho-1-depleted larvae are those of the secondary lineage, and that only primary muscle cells were lost.

To confirm that primary muscle is lost in macho-1-depleted larvae, we isolated and cultured the primary muscle-lineage blastomeres, B4.1, from eight-cell embryos (Fig. 3f–j). In B4.1 partial embryos derived from scrambled and sense oligonucleotide-injected eggs, normal muscle formation was observed after staining with myosin antibody (Fig. 3f). By contrast, injection of antisense oligonucleotide suppressed muscle formation (Fig. 3g). The partial embryos still expressed alkaline phosphatase activity, suggesting



n–r, Embryos at the 110-cell stage fixed and *in situ* hybridized with the muscle actin (*HrMA4*) probe. **n**, Uninjected control. Actin expression is observed in ten precursor blastomeres of primary muscle cells. **o**, macho-1-depleted embryos. **p, q**, Synthesized macho-1 mRNA was subsequently injected. Actin is ectopically expressed in endoderm (**p**) and epidermis (**q**) blastomeres. **r**, Subsequent injection of mutant mRNA has no effect. **s, t**, Eggs injected with 25 pg (**s**) and 125 pg (**t**) macho-1 mRNA without injection of antisense oligonucleotide. Myosin expression is detected. **u**, Formation of ectopic myosin-expressing cells in mRNA-injected partial embryo derived from an a4.2 blastomere. **v, w**, Actin expression in 110-cell embryos injected with 25 pg (**v**) and 125 pg (**w**) macho-1 mRNA. **x, x'**, Immunostaining of Flag epitope in 110-cell embryos injected with wild-type (**x**) and mutant mRNA (**x'**). **y, y'**, Detection of myosin expression in a4.2 partial embryos to which posterior–vegetal cytoplasm of normal egg (**y**) and macho-1 depleted egg (**y'**) was transferred. Scale bars, 100 μm (**a, c, r, t, w**); 50 μm (**j, m, u, x', y'**).

that they were viable and that endoderm formation was not affected (Fig. 3h).

The most reliable way of demonstrating the specificity of the antisense effect is through rescue, by injection of macho-1 mRNA. Therefore, 25 pg of synthesized mRNA was subsequently injected after depletion of endogenous mRNA. The defect of muscle formation was rescued in all cases (Fig. 3i). By contrast, mRNA that lacked the zinc-finger domain (see Fig. 1b) failed to correct the defect (Fig. 3j). Similar results were obtained when muscle formation was examined by detecting AchE expression (Fig. 3k–m). These results are summarized in Fig. 4. When we injected wild-type mRNA, the expression of alkaline phosphatase was significantly lower, most probably as a result of the conversion of endoderm to muscle cells (see Fig. 3p). Mutant mRNA had slight rescuing activity, as some embryos occasionally formed small numbers of muscle cells (Fig. 3m). One explanation could be that endogenous macho-1 mRNA is not completely removed, and antisense oligodeoxynucleotides remain after overnight incubation, thereby suppressing translation from residual messages. Injected mutant mRNA may release the oligodeoxynucleotides from the residual messages, as the mutant mRNA has the sequence that is complementary to the antisense oligonucleotide.

In ascidians, expression of various zygotic genes is initiated during cleavage stages. The expression of the muscle actin gene is an example, being observed in all cells of the primary muscle lineage at the 110-cell stage¹⁰. Its expression in the secondary muscle lineage is not initiated until a later stage of development. Actin mRNA was detected in the ten B-line muscle lineage blastomeres in uninjected and control oligonucleotide-injected embryos (Fig. 3n). In macho-1-deficient embryos, expression of actin was suppressed in most muscle precursors (Fig. 3o). When macho-1 mRNA was subsequently injected, expression in muscle blastomeres was rescued. Furthermore, ectopic expression was observed in endoderm (Fig. 3p) and epidermis (Fig. 3q) precursors in 97% of the cases. Injected mutant mRNA had, at most, residual rescue activity at the 110-cell stage (Fig. 3r). Perhaps more rescuing activity would be detected at later stages. In addition, mutant mRNA had no activity to promote ectopic actin expression.

We next examined the effects of overexpressing macho-1 by

injection of wild-type or mutant mRNAs into fertilized eggs without injection of the antisense oligonucleotide. Whereas injection of mutant mRNA did not affect development, injection of 25 pg wild-type mRNA resulted in aberrant embryogenesis, although a residual head and tail were still recognizable (Fig. 3s). When 125 pg wild-type mRNA was injected, embryos developed into masses of cells in which large amounts of muscle cells seemed to be formed (Fig. 3t). However, in these specimens, it was difficult to determine whether or not ectopic muscle had formed. We therefore carried out blastomere isolation experiments (Fig. 5). Primary muscle is derived from the B4.1 blastomere pair of the eight-cell embryo. The A4.1 and b4.2 blastomeres give rise to secondary muscle, whereas descendants of a4.2 do not make muscle. Each blastomere pair was isolated and allowed to develop into partial embryos. In uninjected and mutant RNA-injected partial embryos, only B4.1 partial embryos developed muscle cells that expressed myosin and AchE. Injection of 25 pg macho-1 mRNA resulted in ectopic expression of the muscle markers in about half of the cases. When 125 pg was injected, progeny from all four isolated blastomeres developed muscle cells in over 90% of the cases (Fig. 3u). We also examined the expression of the actin gene in whole embryos at the 110-cell stage. As in the previous rescue experiment, ectopic actin expression was observed, even at a low dose (Fig. 3v); at a high dose, many blastomeres expressed the actin gene (Fig. 3w).

As synthesized mRNAs have a Flag tag (Fig. 1b), mRNA-injected embryos were immunohistochemically stained with anti-Flag antibody to show the location of the overexpressed protein. In the 110-cell embryos that were injected with wild-type mRNA, the nuclei of blastomeres were stained (Fig. 3x). By contrast, in mutant mRNA-injected embryos, staining was preferentially observed in perinuclear cytoplasm (Fig. 3x'). Thus, both the mutant and wild-type proteins were translated by the 110-cell stage. The result also indicates that macho-1 may be a nuclear protein, which is consistent with its putative function as a transcription factor similar to vertebrate *Zic*⁸. In the ascidian embryo, the expression of actin, myosin and snail genes is initiated as early as the 32-cell stage^{10,15,16}, whereas that of the myogenic factor homologue (*AMD1*) and the *Tbx6* homologue (*AsT-2*) start at the 64-cell stage^{17,18}. It is therefore plausible that macho-1 promotes the expression of these muscle

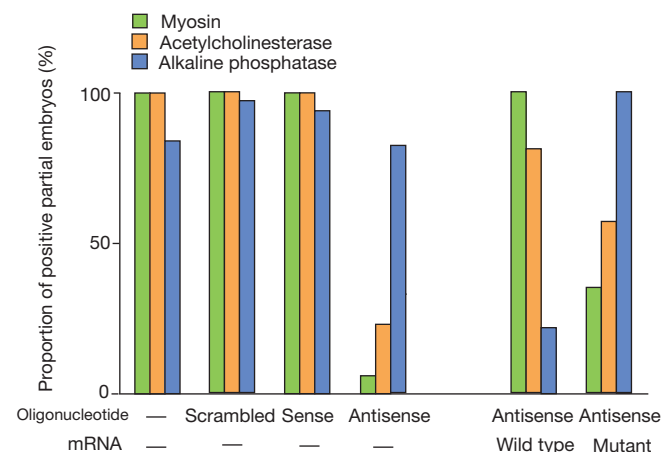


Figure 4 Effect on muscle and endoderm formation after injection of various oligodeoxynucleotides and synthesized mRNA. Scrambled, sense and antisense oligodeoxynucleotides were injected into unfertilized eggs as indicated. Wild-type and mutant mRNAs were subsequently injected after fertilization (right). Precursor blastomeres (B4.1) for primary muscle and endoderm were isolated at the eight-cell stage and allowed to develop until the hatching stage. Partial embryos were stained for the expression of myosin (green), AchE (orange) and endoderm-specific ALP (blue). Each column represents the results of more than 30 specimens.

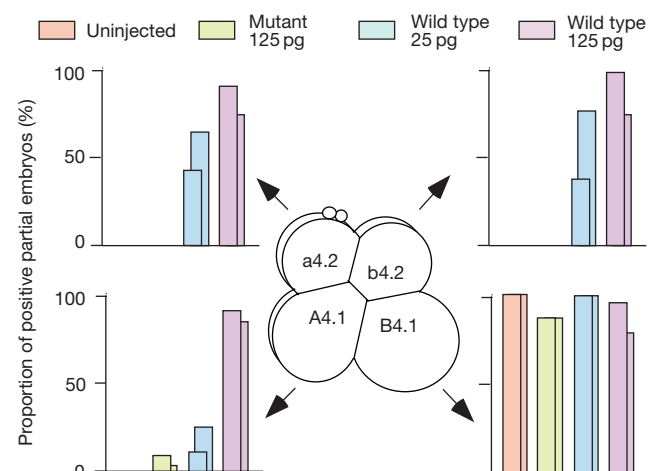


Figure 5 Overexpression of macho-1 mRNA results in ectopic formation of muscle cells. Mutant (125 pg) and wild-type (25 and 125 pg) mRNAs were injected just after fertilization, then each blastomere pair was isolated from the eight-cell embryo (anterior is to the left, animal pole is top). Front and back columns represent the proportion of partial embryos that express myosin and AchE, respectively. Each column represents the results of 30–70 specimens.

structural genes and the regulatory/maintenance factor genes by binding directly to the regulatory elements of these genes, although we do not have direct evidence for such an interaction.

Our results indicate that maternal *macho-1* mRNA may be both required and sufficient for specification of muscle fate during embryogenesis. Furthermore, localization of the mRNA in eggs coincides with that of muscle determinants inferred from cytoplasmic transfer experiments. These criteria, however, are not enough to confirm conclusively that *macho-1* is, in fact, the localized muscle determinant. For example, in the frog egg, β -catenin is required and sufficient for promoting the development of dorsal structures. But when the dorsal cytoplasm of β -catenin-depleted eggs is transferred to the ventral side of an intact egg, a secondary dorsal axis is still induced¹⁹. This observation suggests that β -catenin functions as a component of the machinery transducing the dorsal determinant, but is not the dorsal determinant itself. Accordingly, we carried out similar experiments to test *macho-1*. The posterior-vegetal cytoplasm of fertilized eggs has an ability to promote muscle formation when transferred to a4.2 blastomeres, which normally give rise to epidermis (see Figs 1e and 5). We reconfirmed that myosin-expressing muscle cells were formed ectopically in 85% of a4.2 partial embryos ($n = 20$) after this operation (Fig. 3y). By contrast, the posterior-vegetal cytoplasm of *macho-1*-depleted eggs promoted muscle formation in only 6% of the cases ($n = 18$, $P < 0.001$) (Fig. 3y'). From these results we conclude that the *macho-1* mRNA is a genuine, localized muscle determinant.

The distribution of maternal *macho-1* mRNA coincided well with that of the muscle determinant (Fig. 1c–h). During cleavage, the mRNA is concentrated in a tiny posterior region, the postplasmic region, as is the case for many postplasmic RNAs^{20–23} (Fig. 1i). At the 16-cell stage, four posterior blastomeres (B5.1 and B5.2 bilateral pairs) in the vegetal hemisphere represent the primary muscle lineage, but only the most posterior blastomere (B5.2) pair inherit the *macho-1* mRNA (Fig. 1j). If *macho-1* is the muscle determinant, the protein must already be translated and partitioned into these blastomeres. Further study with antibody against *macho-1* protein is required to show protein localization. At the 110-cell stage, the most posterior (B7.6) cells inherit exclusively the mRNA (Fig. 1k). The blastomeres are no longer muscle lineage cells, but are fated to the endodermal strand. During morphogenesis, maternal mRNAs are retained in the endodermal strand cells (Fig. 1l, m), and are lost at the late tailbud stage. We observed no zygotic expression of the *macho-1* gene.

In conclusion, maternal *macho-1* mRNA satisfies key criteria for the muscle-forming factor in ascidian eggs, whose existence was proposed a century ago⁵. As ascidians are primitive chordates, it will be interesting to investigate the function of *macho-1* homologues in advanced chordates. A number of localized maternal RNAs have been identified in ascidian eggs by subtraction screening^{20,21} and complementary DNA project of maternal mRNA^{22–24}. Our methods for depleting the specific maternal mRNAs will provide a means of identifying their developmental functions. □

Methods

Cloning of cDNA

We devitellinated 400 *H. roretzi* embryos at the eight-cell stage, and bisected them to animal and vegetal hemispheres with a glass needle. Both hemispheres were extracted with detergent and glycerol to recover mRNAs anchored to the cytoskeleton, as described²⁵. mRNA was purified from the extracted samples using a Micro-FastTrack mRNA purification kit (Invitrogen) at 1/10 of the scale. We carried out conversion to cDNA from the mRNA, and PCR-based subtractions as described²⁶. After second strand synthesis, cDNAs derived from the animal and vegetal hemispheres were ligated with P-linker (5'-GATTACTCGAGACTAATATC-3', pGATATTAGTCTCGAGTA-3') and F-linker (5'-TCGACTCGAGTATAGTACA-3', pTGAAGTATAGTCTCGAGT-3'), respectively, and amplified by PCR²⁶. Subtraction was carried out by mixing 10 ng cDNA from the vegetal hemisphere with 1 μ g biotinylated cDNA from the animal hemisphere. Annealed cDNA and an excess amount of biotinylated cDNA were absorbed on Dynabeads M-280 conjugated with streptavidin (Dyna), and the remaining cDNA was amplified with the primers for the F-linker. After three rounds of subtraction and amplification, the resulting

cDNA was used for screening vegetal-specific clones from a fertilized egg cDNA library. We isolated 48 positive clones by standard protocols. *In situ* hybridization showed that three clones represented maternal mRNAs with strong localization at the posterior pole of early embryos. Sequencing of these clones showed that two of them included *macho-1* cDNAs, and that the other had a cDNA of HrPEM (accession number AB045129).

Depletion of mRNA

Antisense oligodeoxynucleotides complementary to two parts of *macho-1* mRNA were synthesized (HPLC grade, Pharmacia Biotech, Japan). These oligodeoxynucleotides depleted *macho-1* mRNA from the eggs in a dose-dependent manner. As one oligonucleotide that covered the starting methionine (5'-G*TT*TT*TCAGATTCCTT C*A*TT*C*G-3' (antisense to a 20-nucleotide sequence spanning nucleotides 39–58 of *macho-1* cDNA)) was more efficient in depleting mRNA from eggs, this was used in the present experiments. The asterisks indicate the phosphorothioate bond. For control experiments, sense and scrambled (5'-T*TT*A*A*CTTGTCGTCG*A*TT*TT*C-3') oligodeoxynucleotides were used. The oligodeoxynucleotides were resuspended in 0.2 M KCl and injected at a dose of 50 pg per egg. Injection of a higher dose resulted in non-specific cleavage retardation and arrest. The method used for depleting maternal mRNAs was modified from that used in *Xenopus* eggs²⁷. Naturally spawned eggs of *H. roretzi* were chemically devitellinated as described²⁸. We injected the unfertilized eggs in seawater containing half the normal Ca concentration to prevent egg activation. Care was taken to avoid sucking seawater into the needle tip because injection of a small amount of seawater would have caused egg activation. Fewer than 20% of the injected eggs were activated, and these activated eggs were discarded. We kept the eggs unfertilized, and incubated them in normal seawater overnight at 11 °C. Eggs without a vitelline membrane are rarely fertilized²⁹. To fertilize them, sperm were preincubated for several minutes with egg seawater, that is, the supernatant of the overnight suspension of vitellinated eggs and seawater in a ratio of 1/1. In addition, sperm were treated with alkaline seawater (pH 10, adjusted with NaOH) to activate motility in some cases. These techniques improved greatly the synchrony of fertilization of devitellinated eggs. Fertilized eggs were reared at 11 °C in the supernatant of a homogenate of cleaving embryos that contained 50 μ g ml⁻¹ streptomycin sulphate and kanamycin sulphate to facilitate normal morphogenesis of devitellinated embryos³⁰. We carried out cytoplasmic transfer experiments as described⁷.

Overexpression of mRNA

Capped RNAs were synthesized by linearizing the plasmid vector (pBSRNT3) containing subcloned cDNAs with *Xho*I, and transcribing the linear template with T3 RNA polymerase using a Megascript kit (Ambion). Wild-type RNA contained the full open reading frame (ORF), whereas the mutant mRNA lacked the zinc-finger domain spanning amino acids 197–369. Synthesized mRNA was suspended in sterile, distilled water and 25 and 125 pg was injected into intact and *macho-1*-depleted eggs after fertilization.

Histochemistry and *in situ* hybridization

Immunofluorescence for *Halocynthia* myosin heavy chain was performed with the Mu-2 antibody¹¹. Histochemistry for AchE and alkaline phosphatase was carried out as described^{9,12}. Flag epitope was detected with anti-Flag M2 monoclonal antibody (Sigma) and secondary antibody conjugated with horseradish peroxidase (SimpleStain MAX PO(M), Nichirei, Tokyo). *In situ* hybridization with the *HrMA4* probe was carried out according to standard protocols¹⁰.

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Adipose-selective targeting of the *GLUT4* gene impairs insulin action in muscle and liver

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The earliest defect in developing type 2 diabetes is insulin resistance^{1,2}, characterized by decreased glucose transport and metabolism in muscle and adipocytes^{3,4}. The glucose transporter GLUT4 mediates insulin-stimulated glucose uptake in adipocytes and muscle by rapidly moving from intracellular storage sites to the plasma membrane⁴. In insulin-resistant states such as obesity and type 2 diabetes, GLUT4 expression is decreased in adipose tissue but preserved in muscle^{3,4}. Because skeletal muscle is the main site of insulin-stimulated glucose uptake, the role of adipose

tissue GLUT4 downregulation in the pathogenesis of insulin resistance and diabetes is unclear. To determine the role of adipose GLUT4 in glucose homeostasis, we used *Cre/loxP* DNA recombination to generate mice with adipose-selective reduction of GLUT4 (*G4A^{-/-}*). Here we show that these mice have normal growth and adipose mass despite markedly impaired insulin-stimulated glucose uptake in adipocytes. Although GLUT4 expression is preserved in muscle, these mice develop insulin resistance in muscle and liver, manifested by decreased biological responses and impaired activation of phosphoinositide-3-OH kinase. *G4A^{-/-}* mice develop glucose intolerance and hyperinsulinaemia. Thus, downregulation of GLUT4 and glucose transport selectively in adipose tissue can cause insulin resistance and thereby increase the risk of developing diabetes.

Mice with GLUT4 deficiency selectively in their adipose tissue (*G4A^{-/-}*) were obtained by crossing mice with a 'floxed' GLUT4 allele (*GLUT4-lox*)^{5,6} with transgenic mice expressing Cre recombinase selectively in adipose tissue. Adipose-specific expression of Cre recombinase was achieved by ligating 5.4 kilobases (kb) of the *aP2* promoter/enhancer⁷ to the *Cre* gene (Fig. 1). Two lines of *aP2-Cre* mice were studied. In both lines, *Cre* expression was restricted to brown adipose tissue (BAT) and white adipose tissue (WAT) (Fig. 1). In line 1, *Cre* expression was greater in BAT than in WAT, whereas in line 2 the reverse was seen. The tissue specificity and high efficiency of *Cre* activity were confirmed by crossing the *aP2-Cre* mice with the *ROSA26-lacZ* reporter mouse^{8,9}, in which a stop codon is flanked by *loxP* sites so *LacZ* is expressed only when a cell is exposed to *Cre*⁹. β -Galactosidase (β -gal) staining of several tissues from *aP2-Cre-ROSA26-lacZ* mice revealed diffuse activation of β -gal expression throughout BAT and WAT, but no activation in skeletal muscle, liver, pancreatic islets, brain or other tissues examined (P. She and M. A. Magnusen, personal communication). *G4A^{-/-}* mice were homozygous for *GLUT4-lox* and hemizygous for the *aP2-Cre* transgene. GLUT4 protein levels were reduced by 70–99% in both BAT and WAT (epididymal, periovarian and subcutaneous) of male and female *G4A^{-/-}* mice (Fig. 2a) generated with either line of *aP2-Cre* mice. In both *G4A^{-/-}* lines, GLUT4 expression was preserved in skeletal muscle and heart (Fig. 2a). GLUT4 expression in adipose tissue of mice expressing *Cre* but no

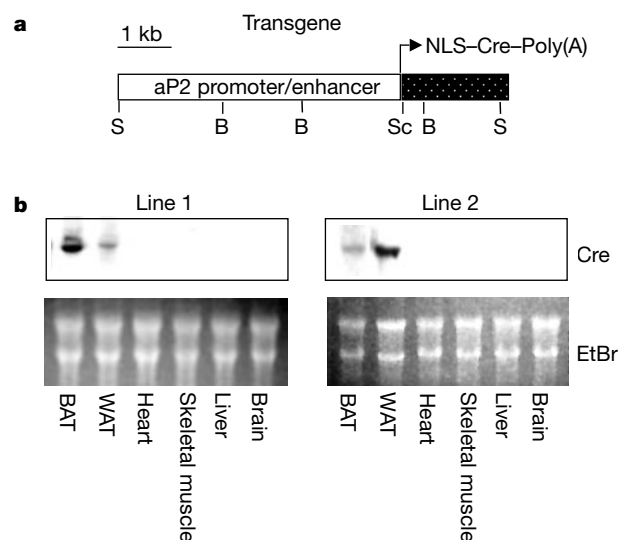


Figure 1 Transgene construct and Cre expression. Representation of *aP2-Cre* transgene (a), and representative northern blots (b) showing that *Cre* expression is limited to brown (BAT) and white (WAT) adipose tissue in two independent lines of transgenic mice. B, *Bam*HI; S, *Sal*I; Sc, *Sac*I restriction sites; EtBr, ethidium-bromide-stained northern gel; NLS, nuclear localization signal.

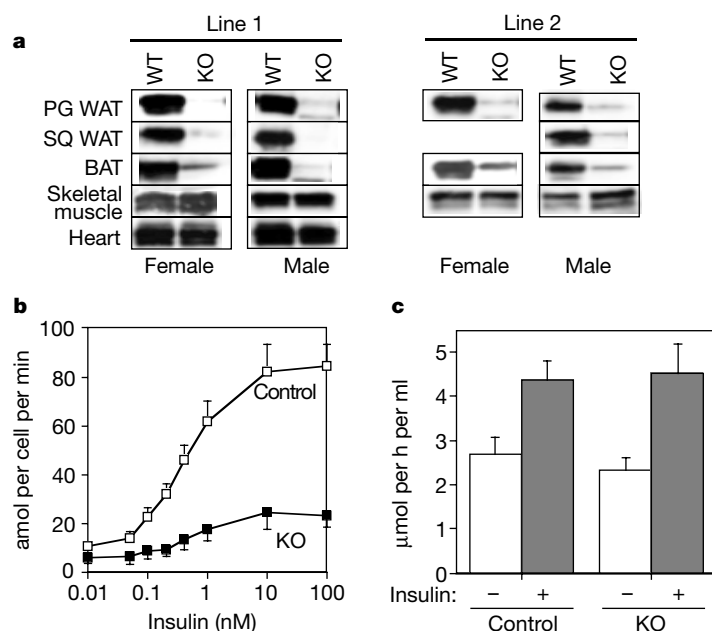


Figure 2 GLUT4 expression and glucose uptake in adipocytes and muscle *ex vivo*. **a**, Representative immunoblots of GLUT4 in perigonadal (PG) and subcutaneous (SQ) white (WAT) and brown (BAT) adipose tissue, gastrocnemius skeletal muscle and heart of wild-type (WT) and $G4A^{-/-}$ (KO) male and female mice from both *aP2-Cre* transgenic lines. Line 1 females and males were 52 and 25 weeks old, respectively; line 2 mice were 12 weeks old. **b**, Dose-response curves for insulin-stimulated glucose uptake in isolated

adipocytes from female $G4A^{-/-}$ (KO) mice ($n = 6$) and control ($n = 12$) littermates. Values at all insulin concentrations are significantly different between $G4A^{-/-}$ and controls ($P < 0.05$). **c**, Basal and insulin-stimulated glucose uptake into isolated EDL muscles of female $G4A^{-/-}$ (KO) ($n = 5$) and wild-type (control) mice ($n = 5$). Similar data were obtained for males (not shown).

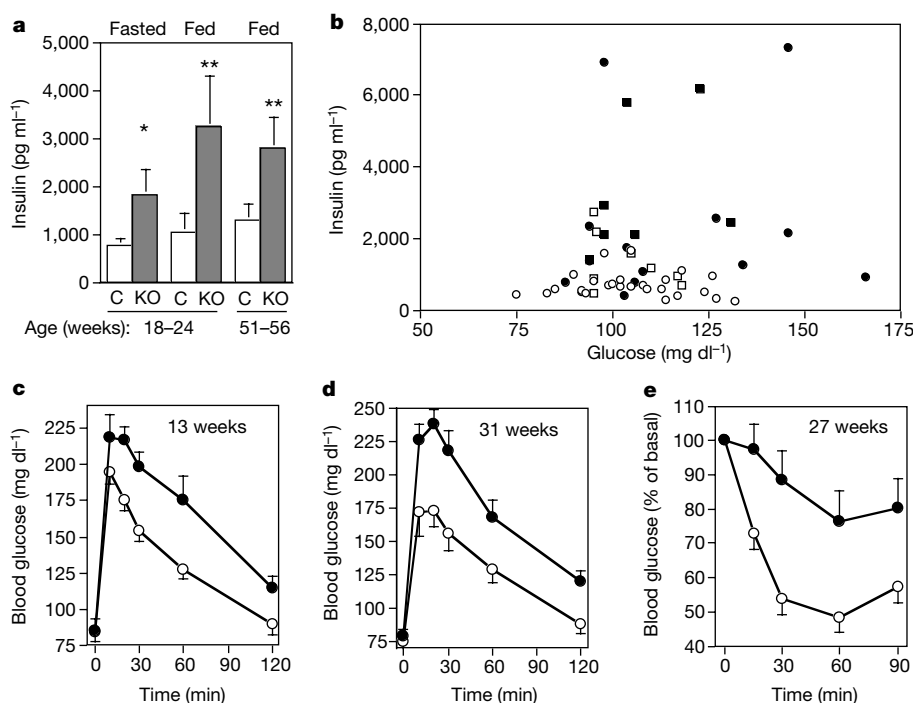


Figure 3 Glucose intolerance and insulin resistance in adipose tissue GLUT4-deficient ($G4A^{-/-}$) mice. **a**, Fasted (12 h) and fed plasma insulin concentrations in control (C, open bars) and $G4A^{-/-}$ (KO, grey bars) female littermates. Data are means $\pm$ s.e.m. of values for 5–12 mice per group. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$ versus controls. **b**, Increased risk of hyperinsulinaemia in $G4A^{-/-}$ mice. Each point shows the fed plasma insulin and glucose levels in an individual female mouse. Open circles, WT ($n = 26$); filled circles, KO ($n = 13$) aged 19–23 weeks. Open squares, WT ($n = 8$); filled squares, KO ($n = 7$) aged 51–56 weeks. **c**, **d**, Glucose tolerance tests (1 mg per kg (body weight) i.p.) in awake female $G4A^{-/-}$ (filled symbols) and wild-type controls (open symbols). **c**, Data from

seven KO and ten WT littermates aged 13 weeks. **d**, Data from seven KO and eight WT mice aged 31 weeks. Glucose values in $G4A^{-/-}$ mice were elevated significantly above controls at all times after glucose injection; $P < 0.001$. **e**, Insulin tolerance tests (0.75 U per kg (body weight) i.p.) in awake female $G4A^{-/-}$ (filled symbols, $n = 9$) and wild-type control (open symbols, $n = 10$) mice at 27 weeks of age. The fall in blood glucose was significantly impaired in $G4A^{-/-}$ mice at all times after insulin injection; $P < 0.001$. Similar results were seen in $G4A^{-/-}$ mice derived from both lines of *aP2-Cre* mice. Results in *lox/lox* (without *Cre*) littermates were indistinguishable from WT and *Cre*^{+/+} controls.

lox was the same as in wild type, but it was slightly reduced in *lox/lox* mice without *Cre* (data not shown). This is possibly due to an effect of the *loxP* sites or *neo* cassette in the GLUT4 gene specifically in adipose tissue as no reduction was seen in skeletal muscle or heart of *lox/lox* mice⁶. *lox/lox* mice were therefore studied as a separate control group. GLUT1 expression was normal in adipose tissue of *G4A^{-/-}* mice (data not shown).

Growth curves were normal in male and female *G4A^{-/-}* mice from 3–16 weeks of age. Body weights, gonadal fat pad weights, and adipocyte size and number were also normal in *G4A^{-/-}* mice at several ages between 16 and 38 weeks. Adipocyte size in μg lipid per cell in females with a mean age of 21 ± 1 weeks was 0.27 ± 0.03 in control ($n = 16$) and 0.25 ± 0.07 in *G4A^{-/-}* ($n = 10$) mice. The number of cells recovered per mg of perigonadal fat was $3,127 \pm 468$ in control and $3,872 \pm 772$ in *G4A^{-/-}* mice. Thus, in contrast to total body ablation of GLUT4 (ref. 10), reduction of GLUT4 selectively in adipose tissue does not result in growth retardation or decreased adipose mass or adipocyte size. Heart weight was also normal in *G4A^{-/-}* mice in contrast to the cardiomegaly observed in *GLUT4* null mice¹⁰ and in mice with cardiac-specific⁵ or muscle-specific⁶ *GLUT4* deletion.

In adipocytes from *G4A^{-/-}* mice, basal glucose uptake tends to be reduced by 40% and insulin-stimulated glucose uptake is markedly blunted at all insulin concentrations, with maximal insulin stimulation reduced by 72% as compared with wild-type adipocytes (Fig. 2b). In contrast, when skeletal muscle of *G4A^{-/-}* mice is studied *ex vivo*, basal and insulin-stimulated glucose transport are normal (Fig. 2c). We studied the extensor digitorum longus (EDL) because it is a glycolytic, fast-twitch muscle representative of the main muscle mass in the mouse¹¹. Thus adipose-specific reduction in GLUT4 causes insulin resistance in adipocytes, whereas insulin action is normal in skeletal muscle *ex vivo*, albeit not *in vivo* (see below).

Fasting and fed glucose concentrations are normal in *G4A^{-/-}* mice. However, in female mice, this requires 2–3-fold higher insulin concentrations (Fig. 3a), indicating significant insulin resistance under ambient conditions. This resistance is seen at the earliest age

studied (16 weeks for line 2; data not shown) and persists to at least 55 weeks. Thirty per cent of *G4A^{-/-}* females have either insulin or glucose concentrations that exceed control levels (Fig. 3b). Twenty per cent of *G4A^{-/-}* females are very insulin resistant, with 3–4 times higher insulin concentrations than the highest concentrations in controls. More than 50% of *G4A^{-/-}* females but only 6% of controls have insulin values exceeding $2,000 \text{ pg ml}^{-1}$. In male *G4A^{-/-}* mice, mean insulin levels are not significantly increased as a wide range in values is seen, but 37% of *G4A^{-/-}* males also have elevated insulin levels which exceed those of controls (data not shown). Thus, adipose selective reduction of GLUT4 predisposes mice to insulin resistance. The variability in glucose and insulin levels in *G4A^{-/-}* mice is typical of outbred strains and reflects the effects of genetic modifiers¹². Glucose tolerance is markedly impaired in female *G4A^{-/-}* mice as early as 13 weeks of age (Fig. 3c) and persists to at least 31 weeks (Fig. 3d).

Insulin resistance was also evident from the blunted fall in blood glucose in *G4A^{-/-}* mice in response to intraperitoneal (i.p.) insulin injection (Fig. 3e). Male *G4A^{-/-}* mice at 12 weeks of age have a similar degree of glucose intolerance and insulin resistance (data not shown). A subset of *G4A^{-/-}* mice develops extreme insulin resistance with flat glucose responses to insulin injection (data not shown). This was seen as early as 12 weeks in *G4A^{-/-}* females and was not seen in any of more than 35 wild-type or *Cre^{+/+}* control mice studied.

We performed hyperinsulinaemic–euglycaemic clamp studies with assessment of 2-deoxyglucose uptake into individual tissues *in vivo*, and measured insulin action on hepatic glucose production. Insulin-stimulated whole body glucose uptake was decreased by 53% in *G4A^{-/-}* mice (Fig. 4a) owing to reductions of ~50 and ~67% in glycolysis and glycogen synthesis, respectively (Fig. 4a). As expected, insulin-stimulated glucose transport into WAT and BAT (Fig. 4b) was markedly reduced *in vivo*. Unexpectedly, glucose transport into skeletal muscle *in vivo* (gastrocnemius, Fig. 4b) was also impaired by ~40% in spite of preserved expression of GLUT4 in muscle (Fig. 2a). As we did not see this impairment in muscle *in vitro* (Fig. 2c), it appears that this defect is secondary to the *in vivo*

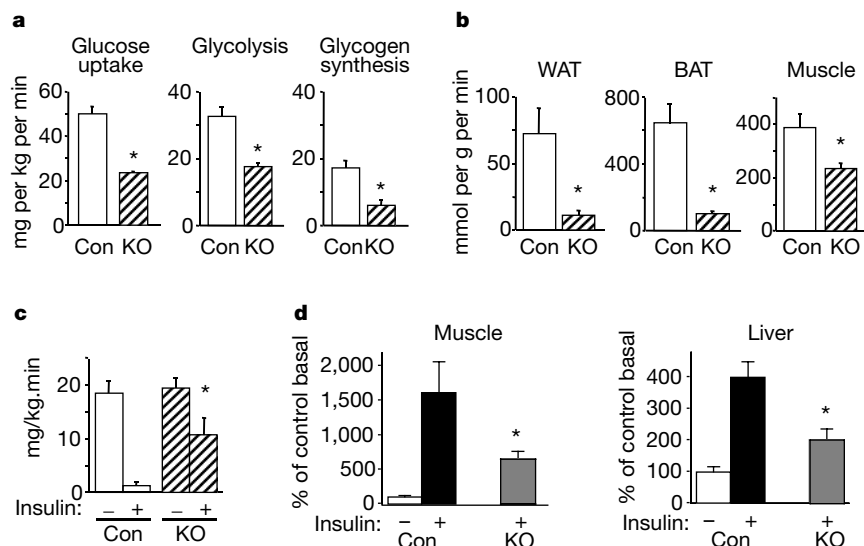


Figure 4 *In vivo* glucose metabolism and PI(3)K activity. **a**, Insulin-stimulated whole body glucose uptake, glycolysis and glycogen synthesis in awake *G4A^{-/-}* females (KO, hatched bars) and wild-type or *Cre^{+/+}* controls (Con, open bars). **b**, Insulin-stimulated 2-deoxyglucose transport *in vivo* in white adipose tissue (WAT), brown adipose tissue (BAT) and gastrocnemius muscle in *G4A^{-/-}* females (hatched bars) and controls (open bars). **c**, Basal and insulin-stimulated rates of hepatic glucose production in *G4A^{-/-}* females (hatched bars) and controls (open bars). Values are means $\pm$ s.e.m. for 5–6

mice per genotype. Asterisk, $P < 0.05$ versus control. **d**, PI(3)K activity measured in phosphotyrosine immunoprecipitates²⁴ prepared from gastrocnemius and liver from fasted (16–18 h), 9–10-month-old male mice. Tissues were collected 3 min after intravenous saline (–) or insulin (+, 10 U per kg (body weight)) injection. Data are means $\pm$ s.e.m. for four saline controls (wild-type and *Cre^{+/+}*) and 8–9 insulin-stimulated mice per group. Asterisk, $P < 0.05$ versus insulin-stimulated control.

milieu and may result from altered release of specific molecules from fat. Hepatic glucose production was normal in the basal state but the ability of insulin to suppress hepatic glucose production was very impaired in $G4A^{-/-}$ mice (Fig. 4c). Insulin resistance was also present at the level of activation of phosphoinositide-3-OH kinase (PI(3)K), a signalling step that is necessary for many metabolic effects of insulin. Insulin-stimulated PI(3)K activity was reduced by 50–60% in muscle and liver (Fig. 4d). This impairment in PI(3)K activation might be a major mechanism underlying the defective responses of muscle and liver to insulin in $G4A^{-/-}$ mice. These data suggest that markedly reduced insulin-stimulated glucose transport in adipocytes secondarily induces insulin resistance in other insulin target tissues.

Known mechanisms by which adipocytes can indirectly regulate insulin action and substrate metabolism in muscle and liver, and insulin secretion from pancreatic β -cells, include release of free fatty acid (FFA)¹³, leptin^{14,15} and tumour necrosis factor (TNF)- α ¹⁶. FFA elevation may cause insulin resistance by impairing insulin signalling through PI(3)K^{17,18}. Reduced glucose transport into adipocytes might limit glycerol synthesis and impair re-esterification of fatty acids into triglyceride, resulting in increased serum FFAs. However, serum FFA levels in the fed and fasted states are normal in $G4A^{-/-}$ mice. Furthermore, FFA levels suppress normally in response to i.p. insulin injection (Fig. 5a), indicating that the insulin resistance in $G4A^{-/-}$ is relatively selective for glucose uptake. Insulin resistance was not associated with dyslipidaemia as plasma triglyceride concentrations were normal (data not shown). The insulin resistance was also not due to increased triglyceride deposition in muscle and liver because the triglyceride content of muscle (11.32 ± 2.19 in $G4A^{-/-}$ versus $13.19 \pm 2.04 \mu\text{mol}$ of lipid per g tissue in controls) and liver (22.83 ± 2.54 in $G4A^{-/-}$ versus 22.61 ± 3.43 in controls) was not increased.

Glucose uptake in rat adipocytes *in vitro* has been suggested to regulate leptin secretion¹⁹ and reduced plasma leptin concentrations may result in insulin resistance²⁰. However, mean plasma leptin levels are normal in $G4A^{-/-}$ mice and plasma leptin concentrations show the same linear relationship with body weight in $G4A^{-/-}$ mice and control littermates (Fig. 5b). Thus, normal glucose uptake in adipocytes is not necessary to maintain normal plasma leptin levels. Elevated TNF- α is another possible mechanism for insulin resistance, but we found no elevation of TNF- α messenger RNA in $G4A^{-/-}$ WAT or BAT (data not shown). Serum TNF- α concentrations were below the level of detection of the mouse enzyme-linked immunosorbent assay (ELISA) in most control and $G4A^{-/-}$ mice. There was no increase in the percentage of $G4A^{-/-}$ mice with

detectable levels, as has been reported in one obese, diabetic mouse model¹⁶. Thus, the insulin resistance in muscle and liver of $G4A^{-/-}$ mice is likely to be caused by effects of chronic hyperinsulinaemia or by changes in the release of an as yet unidentified adipocyte-derived molecule that affects insulin action in other tissues.

In summary, adipose-selective depletion of GLUT4 in mice leads to impaired glucose tolerance and insulin resistance with preserved adipose mass. Insulin resistance occurs secondarily in muscle and liver as evident from defective proximal signalling and reduced biological responses. The insulin resistance cannot be accounted for by changes in circulating FFAs, triglycerides or leptin, or changes in TNF- α expression in adipose tissue. The degree of glucose intolerance and insulin resistance in $G4A^{-/-}$ mice is similar to that in mice with muscle-selective ablation of GLUT4 (ref. 6), thereby suggesting distinct and complementary roles for adipose tissue and skeletal muscle in mediating glucose disposal *in vivo*. Thus, glucose transport in adipose tissue plays a critical role in glucose homeostasis and the adipose-selective downregulation of GLUT4 seen in human obesity and type 2 diabetes may contribute to insulin resistance and to the risk of developing type 2 diabetes. □

Methods

Mice

The $aP2$ -Cre transgene was made by cloning a 1.4-kb *SacI/SalI* complementary DNA fragment encoding Cre recombinase, modified by inclusion of a nuclear localization sequence (NLS) (a gift from K. Rajewsky) and containing a consensus polyadenylation signal, immediately downstream of the 5.4-kb promoter/enhancer of the fatty-acid-binding protein $aP2$ (a gift from B. Spiegelman) (Fig. 1). After injection into male pronuclei of mouse zygotes of the FVB strain, two independent lines of transgenic mice with adipose-selective Cre expression were obtained. Transgenic mice were identified by Southern blotting (*Bam*HI digest of genomic DNA probed with Cre cDNA) or by PCR⁵. Tissue-specific excision was verified by breeding the $aP2$ -Cre mice to mice carrying a *ROSA26-lacZ* gene in which a stop codon in the promoter is floxed⁹.

$G4A^{-/-}$ mice were derived by crossing *GLUT4 loxP* heterozygous mice ($GLUT4^{+/lox}$) with $GLUT4^{4/+lox}$ mice that also expressed Cre recombinase under the control of the $aP2$ promoter/enhancer ($aP2Cre-GLUT4^{4/+lox}$). $G4A^{-/-}$ mice have the genotype *Cre lox/lox*. The remaining genotypes, $+/+$ (wild type), $+/lox$ and lox/lox (all without Cre) and $Cre^{+/+}$, have similar metabolic characteristics. $G4A^{-/-}$ mice were born with the expected mendelian frequency. Thus absence of adipose GLUT4 caused no embryonic lethality. The Institutional Animal Care and Use Committee (Beth Israel Deaconess Medical Center, Boston, MA) approved all studies. Animals were maintained on a 12-hour light/dark schedule (light on at 0600) and had free access to laboratory chow and water.

Immunoblots

Post-nuclear membranes prepared from cardiac muscle and adipose tissue were immunoblotted for GLUT1 and GLUT4 (ref. 5). Gastrocnemius muscles were homogenized using a medium Polytron setting in buffer (HEPES 50 mM, Triton X 1%, sodium pyrophosphate 50 mM, NaF 100 mM, EDTA 10 mM, vanadate 10 mM, aprotinin $10 \mu\text{g ml}^{-1}$, leupeptin $10 \mu\text{g ml}^{-1}$, benzamide 2 mM). After initial centrifugation at 13,000g, the supernatant was then centrifuged at 100,000g for 1 h at 4°C. The resulting supernatant was used for immunoblotting⁵.

Adipocyte size, number and glucose transport *ex vivo*

Adipocytes were isolated from periovarian or epididymal fat pads from fed mice by collagenase (1 mg ml^{-1}) digestion and glucose transport was measured²¹. Aliquots of adipocytes were fixed with osmic acid and counted in a Coulter counter²². Adipocyte mass was determined by dividing the lipid content of the cell suspension by the cell number²².

Skeletal muscle glucose transport

After an overnight fast, mice were killed by CO_2 inhalation, EDL muscles were rapidly dissected and glucose transport was measured⁶.

Metabolite assays

Blood glucose was measured with a One Touch II glucose meter (Lifescan; Johnson & Johnson, Milpitas, CA). Plasma insulin was measured with the Rat Insulin Elisa Kit (ChrysalChem, Chicago, IL) using rat standards. Serum free fatty acids were measured using the NEFA-C kit (Wako Chemicals GMBH, Neuss, Germany) with oleic acid as the standard. Plasma triglycerides and glycerol were measured using the GPO-Trinder colorimetric assay kit (Sigma). Plasma leptin was measured using the Rat Leptin RIA kit (Linco Research, St Louis, MO).

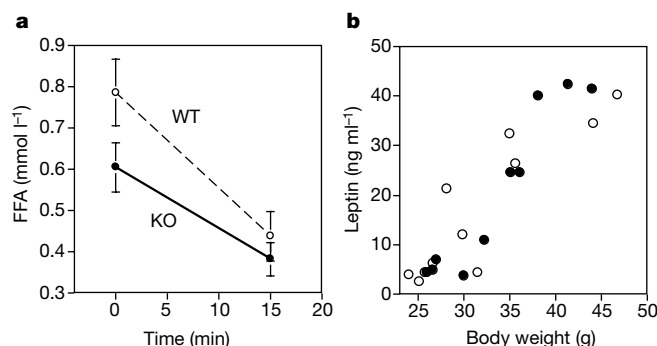


Figure 5 Free fatty-acid (FFA) responses and leptin levels in female $G4A^{-/-}$ mice. **a**, FFA levels were measured before and 15-min after the administration of insulin during the ITT (see Fig. 3). Insulin suppressed FFAs to a similar degree in KO (filled circles) and WT (open circles) mice. **b**, Fed leptin concentrations in $G4A^{-/-}$ ($n = 10$, filled circles) and wild-type littermate controls ($n = 11$, open circles), as a function of body weight (mean age 50 ± 2.5 weeks).

Glucose and insulin tolerance tests

Glucose tolerance tests were performed in awake mice after a 12-h fast⁵. Insulin tolerance tests were performed in awake mice after a 6-h fast⁶.

Whole-body, skeletal muscle and liver glucose flux *in vivo*

Hyperinsulinaemic–euglycaemic clamp studies with uptake of [¹⁴C]2-deoxyglucose into individual tissues were performed as described²³ in awake female mice at 6 months of age. Insulin was infused continuously for 120 min at 2.5 mU per kg (body weight) per min. Basal and insulin-stimulated rates of glucose turnover were measured with continuous [3-³H]glucose infusion.

Phosphoinositide-3-OH kinase activity

Mice were fasted for 16–18 h, injected i.v. with saline or insulin (10 U per kg (body weight)) and killed 3 min after injection. Tissues were collected and frozen. PI(3)K activity was measured in phosphotyrosine immunoprecipitates (monoclonal antibody PY99, Santa Cruz Biotechnology, Santa Cruz, CA) from muscle and liver lysates as described²⁴.

Tissue triglyceride content

Triglyceride content in quadriceps muscle and liver was determined as described²⁵.

Adipocyte TNF- α mRNA and serum TNF- α levels

RNA was extracted from WAT and BAT using Trizol (GibcoBRL) and the expression of TNF- α mRNA relative to GAPDH mRNA or 18S ribosomal RNA was determined by real-time PCR (TaqMan, PE Systems). Serum TNF- α levels were determined in serum samples (50 μ l) using the mouse TNF- α ELISA (Endogen). Both mRNA and serum levels were assessed in age- and sex-matched male and female mice at 12–13 and 26 weeks of age.

Statistical analysis

Data are expressed as mean $\pm$ s.e.m. Differences between two groups were assessed using the unpaired two-tailed *t*-test and among more than two groups by analysis of variance (ANOVA). Data involving more than two repeated measures (glucose and insulin tolerance tests) were assessed by repeated measures ANOVA. Analyses were performed using Statview Software (BrainPower, Calabasas, CA).

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Negative regulation of T-cell activation and autoimmunity by *Mgat5* N-glycosylation

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T-cell activation requires clustering of a threshold number of T-cell receptors (TCRs) at the site of antigen presentation, a number that is reduced by CD28 co-receptor recruitment of signalling proteins to TCRs^{1–5}. Here we demonstrate that a deficiency in β 1,6 N-acetylglucosaminyltransferase V (*Mgat5*), an enzyme in the N-glycosylation pathway, lowers T-cell activation thresholds by directly enhancing TCR clustering. *Mgat5*-deficient mice showed kidney autoimmune disease, enhanced delayed-type hypersensitivity, and increased susceptibility to experimental autoimmune encephalomyelitis. Recruitment of TCRs to agonist-coated beads, TCR signalling, actin microfilament re-organization, and agonist-induced proliferation were all enhanced in *Mgat5*^{−/−} T cells. *Mgat5* initiates GlcNAc β 1,6 branching on N-glycans, thereby increasing N-acetylglucosamine⁶, the ligand for galectins^{7,8}, which are proteins known to modulate T-cell proliferation and apoptosis^{9,10}. Indeed, galectin-3 was associated with the TCR complex at the cell surface, an interaction dependent on *Mgat5*. Pre-treatment of wild-type T cells with lactose to compete for galectin binding produced a phenocopy of *Mgat5*^{−/−} TCR clustering. These data indicate that a galectin–glycoprotein lattice strengthened by *Mgat5*-modified glycans restricts TCR recruitment to the site of antigen presentation. Dysregulation of *Mgat5* in humans may increase susceptibility to autoimmune diseases, such as multiple sclerosis.

Specific glycan structures regulate lymphocyte adhesion, re-circulation and maturation, as demonstrated by the GDP-fucose deficiency in LADII patients¹¹, and immune defects associated with C2 N-acetylglucosamine (GlcNAc)-T(L)¹² or ST3Gal-I (ref. 13)

mutant mice. Depletion of the Mgat5-modified glycans by swainsonine, an inhibitor of α -mannosidase II, potentiates antigen-dependent T-cell proliferation¹⁴. Mgat5 catalyses the addition of β 1,6-GlcNAc to *N*-glycan intermediates found on newly synthesized glycoproteins that transit the medial Golgi¹⁵ (Fig. 1a). The glycans are elongated in *trans*-Golgi to produce tri (2,2,6) and tetra (2,4,2,6) antennary *N*-glycans, which are extended with *N*-acetyl-lactosamine (Gal- β 1,4-GlcNAc) and polymeric forms of *N*-acetyl-lactosamine⁶. To further explore the function of Mgat5 in T-cell immunity, we examined Mgat5-deficient mice for evidence of immune dysfunction. *Mgat5*^{-/-} mice are born healthy, and lack Mgat5 *N*-glycan products in all tissues examined¹⁶. At 3 months of age, peripheral white blood cells, erythrocyte and serum levels of immunoglobulin (Ig)M and IgG were comparable in *Mgat5*^{-/-}, *Mgat5*^{+/-} and *Mgat5*^{+/+} mice (data not shown). The CD4 and CD8 reactive T-cell populations in the spleen and thymus were also in the normal range (Fig. 1b, c). At 12–20 months of age, an increased incidence of leukocyte colonies in kidney and enlarged spleens was observed in *Mgat5*^{-/-} mice. Furthermore, 32% of the *Mgat5*^{-/-} (6 out of 19 mice) had macroscopic haematuria, mononuclear infiltrates and extensive accumulation of fibrin within Bowman's space, characteristic of proliferative glomerulonephritis (Fig. 1d). This form of renal injury is often observed in autoimmune-mediated glomerulonephritis. Milder renal defects were observed in 68% of the *Mgat5*^{-/-} mice but not in the *Mgat5*^{+/-} or *Mgat5*^{+/+} mice.

To examine T-cell responses in the mice, we induced a type IV delayed-type hypersensitivity (DTH) reaction, and measured tissue swelling. The protein-reactive hapten oxazolone was applied topically to the backs of the mice, then again 4 d later to the right ear. Ear swelling in *Mgat5*^{+/+} mice peaked 24 h after application, and swelling was completely gone by day 5. Ear swelling in *Mgat5*^{-/-} mice attained a higher maximum between 48 and 72 h, and persisted for a longer time (Fig. 1e). To study T-cell-dependent autoimmunity *in vivo*¹⁷, we induced experimental autoimmune encephalomyelitis (EAE) by immunizing mice with myelin basic protein (MBP) at three doses (25, 100 and 500 μ g per mouse). At the lowest dose of MBP, the incidence of EAE was significantly greater in *Mgat5*-deficient mice. Furthermore, 25 and 100 μ g doses of MBP produced more severe EAE in *Mgat5*^{-/-} mice compared with wild-type littermates, characterized by an earlier onset, greater motor weakness and more days with disease (Table 1). Myelin injections of 500 μ g induced disease in all mice with greater peak scores and no significant differences in disease incidence or severity between genotypes. These results indicate that mice lacking Mgat5-modified glycans are more susceptible to DTH and EAE autoimmune disease.

In vitro, splenic T cells from *Mgat5*^{-/-} mice hyperproliferated in response to anti-TCR α/β antibody (Fig. 2a). To examine this hypersensitivity in more detail, purified *ex vivo* T cells were cultured at low density and stimulated with increasing concentrations of soluble anti-CD3 ϵ antibody in the presence or absence of anti-CD28

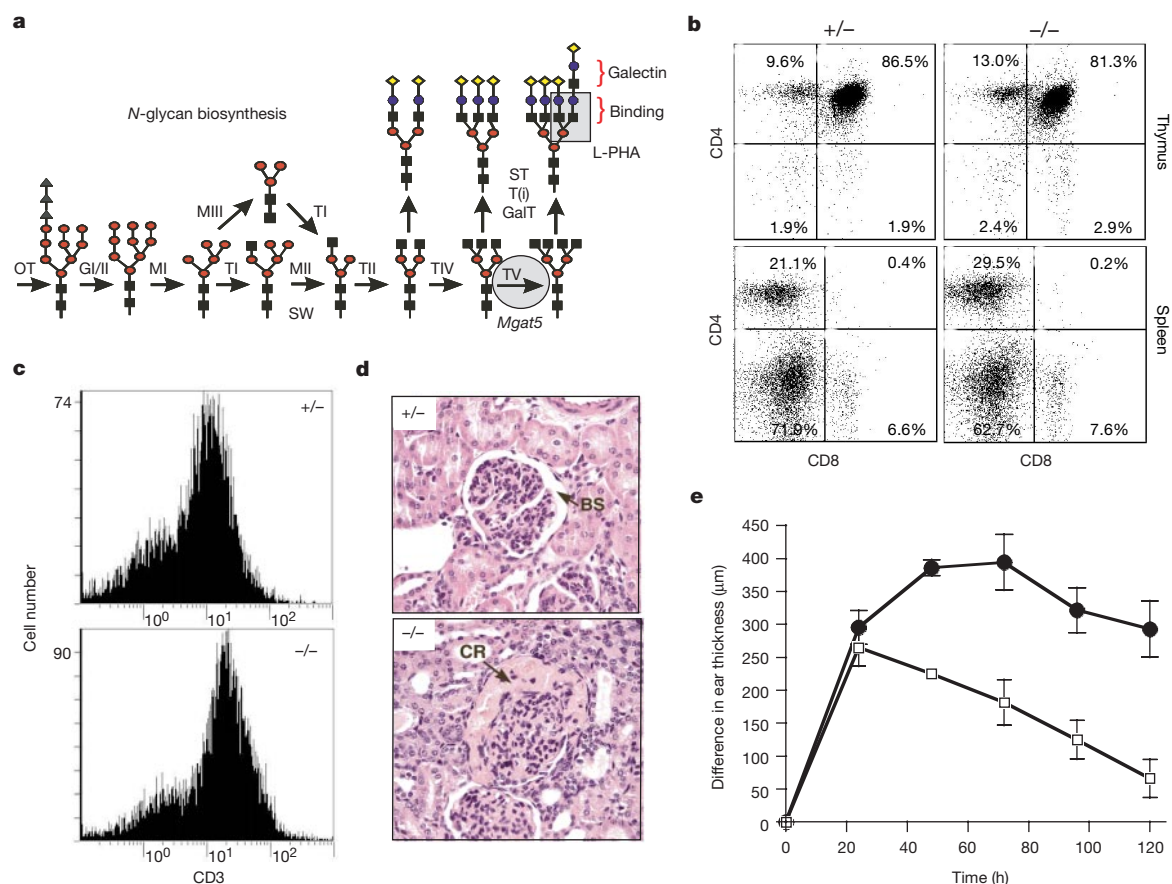


Figure 1 Immune phenotype in *Mgat5*^{-/-} mice. **a**, The Golgi *N*-glycan biosynthesis pathway shows Mgat5 (TV) in the production of a tetra (2,4,2,6) antennary (numbers refer to the linkages of the antennae from left to right). OT, oligosaccharyltransferase; Gl, GII, the α -glucosidases; TI, TII, TIV, TV, T(i), the β -*N*-acetylglucosaminyltransferases; MI, α 1,3/6mannosidases MII, MIII, the α 1,2mannosidases; Gal-T, β 1,4-galactosyltransferases; ST, α -sialyltransferases; and SW, swainsonine block. The boxed structure Gal- β 1,4-GlcNAc- β 1,6(Gal- β 1,4-GlcNAc- β 1,2)Man α binds L-PHA. The galectin-binding disaccharide *N*-acetyl-lactosamine (Gal- β 1,4-GlcNAc) is present in all antennae, and units are marked with red brackets in polylactosamine. **b**, Distribution of CD4⁺ and CD8⁺ cells

in spleen and thymus by FACS analysis using FITC- or phycoerythrin-conjugated antibodies (Pharmingen) reactive to CD3 ϵ , CD4 and CD8. **c**, TCR complex staining of spleen cells by FITC-anti-CD3 ϵ antibodies and FACS analysis. **d**, Light microscopy of kidney showing crescentic glomerulonephritis with a large crescent (CR) of mononuclear cells and fibrin obliterating the Bowman's space (BS) in *Mgat5*^{-/-} mice. **e**, DTH inflammatory response in *Mgat5*^{-/-} (circles) and *Mgat5*^{+/+} (squares) mice exposed to oxazolone (see Methods). The results are plotted as mean change $\pm$ standard error in ear thickness relative to the vehicle-treated left ear for seven *Mgat5*^{-/-} and six *Mgat5*^{+/+} control littermates. $P < 0.01$ with a student *t*-test comparing the genotypes at 2–5 d.

Table 1 Clinical observations of experimental autoimmune encephalomyelitis

Groups (dose)	Incidence of EAE	Peak score	Onset (days)	Days with disease	Deaths
<i>Mgat5</i> ^{+/+} (25 µg)	3/11	0.45 ± 0.24	24.0 ± 3.9	7.0 ± 3.9	0
<i>Mgat5</i> ^{-/-} (25 µg)	9/11*	1.82 ± 0.39†	19.8 ± 3.3†	11.5 ± 3.0†	1
<i>Mgat5</i> ^{+/+} (100 µg)	10/10	1.6 ± 0.22	25.0 ± 2.2	18.5 ± 2.2	0
<i>Mgat5</i> ^{-/-} (100 µg)	10/10	2.1 ± 0.34†	17.6 ± 2.9†	23.3 ± 3.5†	1
<i>Mgat5</i> ^{+/+} (500 µg)	12/12	3.0 ± 0.43	8.9 ± 1.2	27.9 ± 4.0	3
<i>Mgat5</i> ^{-/-} (500 µg)	12/12	2.83 ± 0.38	9.3 ± 0.95	27.2 ± 2.9	2

Disease severity was scored on a scale of 0–5 with: 0, no illness; 1, limp tail; 2, limp tail and hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness/paralysis and hindlimb paralysis; and 5, moribundity or death. Means ± standard error of incidence, peak score and days with disease were calculated using the total number of mice injected per dose as the denominator. The mean ± standard error for day of onset was determined by only using those mice that developed diseases. *Contingency test, $P < 0.001$. †Mann–Whitney U -test comparing genotypes for significant differences at $P < 0.05$.

antibody (Fig. 2b). Both the *Mgat5* deficiency and CD28 engagement reduced the requirements for TCR agonist as indicated by D_{50} values, and were additive when combined (Fig. 2c). Furthermore, the apparent Hill coefficient (n_H), a measure of synchrony in the responding cell population, was increased by both the *Mgat5* deficiency and by CD28 engagement. Therefore, the stimulatory effects of the *Mgat5* mutation and CD28 co-receptor engagement were additive and similar in potency.

Alterations in cell-surface TCR complex levels and intracellular signalling potential of T cells were examined and discounted as possible causes of the *Mgat5*^{-/-} hypersensitivity. The *Mgat5* deficiency did not significantly alter cell-surface expression of CD3, CD4, CD8, TCRα/β, CD28 or CTLA-4 glycoproteins in resting T cells (Fig. 1b, c; and data not shown). Intracellular signalling potential in *Mgat5*^{-/-} T cells is normal, as treatment with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the Ca^{2+} ionophore ionomycin stimulated T cells equally well from mice of both genotypes (Fig. 2d).

We next examined the relationship between cell-surface *Mgat5*-

modified glycans and T-cell activation. Leukoagglutinin (L-PHA) is a tetravalent plant lectin and is a commonly used T cell mitogen that binds specifically to *Mgat5*-modified glycans. *Mgat5*^{-/-} T cells were completely unresponsive to L-PHA, confirming that *Mgat5*-modified glycans are required for stimulation by this lectin (Fig. 2e). L-PHA reactive *N*-glycans are also present on B cells, but L-PHA is not a B cell mitogen. Furthermore, B-cell responses to anti-IgM antibody, lipopolysaccharide and interleukin (IL)-4 plus anti-CD40 antibody were similar for cells from *Mgat5*^{-/-} and *Mgat5*^{+/+} mice (Fig. 2f; and data not shown). In T cells, L-PHA induces signalling common to TCR engagement, including phosphorylation of CD3ζ, Ca^{2+} mobilization, PKC-γ and Ras/mitogen-activated protein kinase (Mapk) activation^{18,19}. The TCRα/β chains have seven *N*-glycans in total, and some are branched, complex-type structures with L-PHA reactivity^{20,21}. These data indicate that *Mgat5*-modified glycans are present on glycoproteins of the TCR complex and are required for L-PHA mitogenesis.

When bound to major histocompatibility complex (MHC)/peptide, TCRs cluster with an inherent affinity greater than unligated TCRs, and the stability of these clusters is critical for intracellular signalling²². However, the density of TCRs measured at the site of T cell–APC (antigen-presenting cell) contact is only marginally increased relative to the remaining cell surface, leaving most of the TCRs unengaged by MHC/peptide⁴. It is possible that ligand-induced TCR clustering in the plane of the membrane may be increased in the absence of *Mgat5*-modified glycans, thus lowering *Mgat5*^{-/-} T-cell activation thresholds. Therefore, to visualize TCR re-organization in response to an antigen-presenting surface, we coated polystyrene beads with anti-CD3ε antibody and incubated them with purified *ex vivo* T cells. After 10 min of contact, TCRs in *Mgat5*^{-/-} cells were markedly more concentrated at the bead surface compared with *Mgat5*^{+/+} cells (Fig. 3a, b). TCRs on wild-type cells could not be induced to cluster to the same extent as *Mgat5*^{-/-} cells, even with longer incubations (20 min) or using anti-CD3ε plus

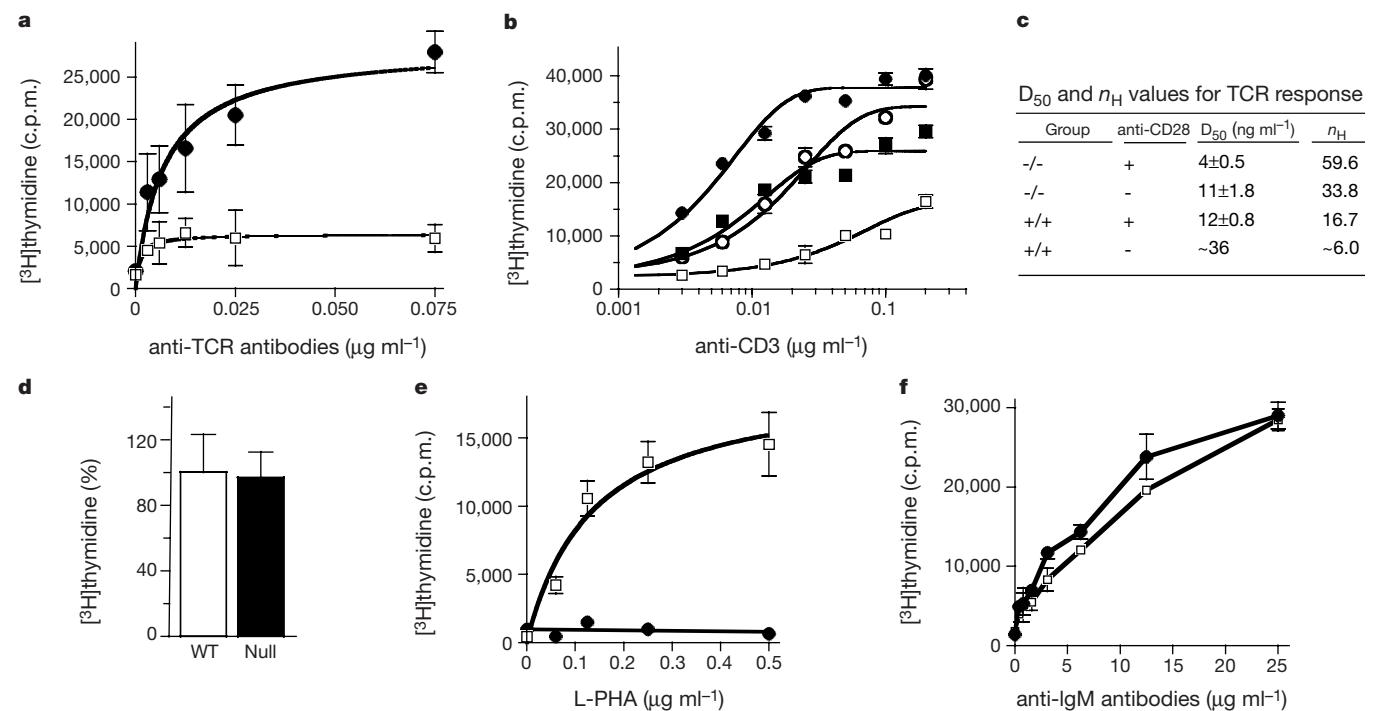


Figure 2 *Mgat5*^{-/-} T cells are hypersensitive to TCR agonists. **a**, Spleen cells were cultured with anti TCRα/β antibodies for 48 h. Filled circles, *Mgat5*^{-/-}; open squares, *Mgat5*^{+/+}. **b**, Purified T cells from spleen were stimulated for 48 h with anti-CD3ε antibody in the absence (open circles and squares) or presence (filled squares and circles) of anti-CD28 antibody. *Mgat5*^{-/-} and *Mgat5*^{+/+} cells are indicated by circles and squares, respectively. **c**, The Hill slope (n_H) of the sigmoidal curve is calculated using

$Y = x^{n_H} / (k^{n_H} + x^{n_H})$. **d**, Stimulation of splenic T cells with TPA plus ionomycin for 48 h. **e**, Stimulation of splenic T cells from *Mgat5*^{-/-} (filled circles) and *Mgat5*^{+/+} (open squares) mice with L-PHA. **f**, Stimulation of splenic B cells from *Mgat5*^{-/-} (filled circles) and *Mgat5*^{+/+} (open squares) mice with anti-IgM antibody for 48 h. The means ± standard deviation of triplicate determinations are used.

anti-CD28-coated beads (data not shown). Actin microfilaments were more concentrated at the bead contact site in *Mgat5*^{-/-} cells, and overlapped more extensively with TCRs in the merged images compared with *Mgat5*^{+/+} T cells (Fig. 3a, b). TCRs are internalized after productive TCR clustering¹, and this was significantly greater in *Mgat5*^{-/-} compared with *Mgat5*^{+/+} cells (Fig. 3c, solid lines). Intracellular signalling mediated by TPA treatment induces TCR internalization but at similar rates in *Mgat5*^{-/-} and *Mgat5*^{+/+} cells (Fig. 3c, dotted lines). Microfilament reorganization was more rapid in *Mgat5*-deficient T cells after soluble anti-CD3ε antibody stimulation (Fig. 3d). Akt/protein kinase B (PKB) phosphorylation is dependent on phosphoinositide 3-OH kinase activity, which stimulates Rac/CDC42 GTPases and actin filament re-

organization²³. Phosphorylated Akt/PKB showed a greater fold increase in *Mgat5*^{-/-} compared with *Mgat5*^{+/+} T cells (Fig. 3d). Mobilization of intracellular Ca²⁺ after stimulation with soluble anti-CD3ε antibody was enhanced in the absence of *Mgat5*-modified glycans (Fig. 3e). Tyrosine phosphorylation of multiple proteins was increased and persisted longer in *Mgat5*^{-/-} T cells exposed to anti-CD3ε antibody coated beads. (Fig. 3e). Immunoprecipitation of Zap-70 revealed increased phosphorylation in *Mgat5*^{-/-} cells 1–5 min after stimulation. Zap-70 kinase binds to dual phosphorylated immunoreceptor tyrosine-based activation motif domains of CD3ζ, and association of the latter with Zap70 was increased in *Mgat5*^{-/-} compared with *Mgat5*^{+/+} T cells (Fig. 3g). Thus, the *Mgat5* deficiency enhanced ligand-dependent TCR aggregation, and

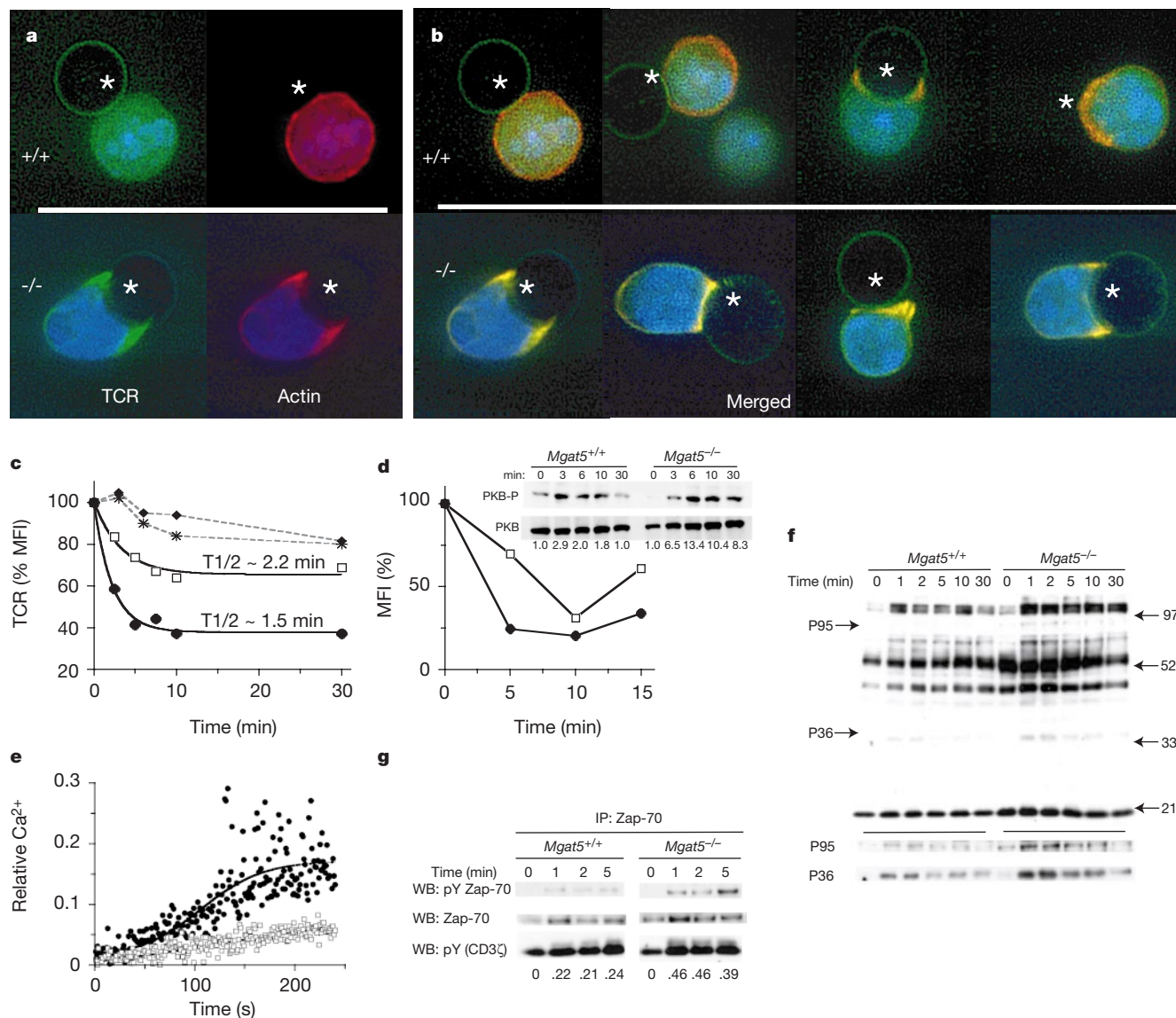


Figure 3 TCR clustering, actin re-organization and signalling in T cells from *Mgat5*^{-/-} and *Mgat5*^{+/+} mice. **a**, TCR and actin microfilament distribution in T cells stimulated by anti-CD3ε-coated beads. **b**, Merged images of *Mgat5*^{-/-} and *Mgat5*^{+/+} cells. Clustering was observed in 5 of 6 and 0 of 6 randomly photographed cells, respectively. **c**, TCR internalization by FACS analysis using FITC-anti-TCRα/β antibodies to measure cell-surface TCRs after addition of anti-CD3ε antibody. Changes in MFI with time are shown. T cells from *Mgat5*^{-/-} (filled circles and squares) or *Mgat5*^{+/+} (open squares and asterisks) mice were treated with anti-CD3ε antibody (filled circles (*Mgat5*^{-/-}) and open squares (*Mgat5*^{+/+})); or with TPA (filled squares (*Mgat5*^{-/-}) and asterisks (*Mgat5*^{+/+})). Similar results were obtained when the stimulation and detection roles of anti-TCRα/β and anti-CD3 were reversed. **d**, Actin polymer content in T cells from *Mgat5*^{-/-} (filled circles), or

Mgat5^{+/+} (open squares) mice after stimulation with anti-CD3ε antibody, measured by FACS. Western blot for phospho-Akt/PKB in T-cell lysates after addition of anti-CDε antibody is shown (top). The values below are fold increase in PKB-P normalized to PKB protein. **e**, Ca²⁺ mobilization in purified T cells from *Mgat5*^{-/-} (filled circles), and *Mgat5*^{+/+} (open squares) mice stimulated with anti-CD3ε antibody. **f**, Western blot with anti-phosphotyrosine antibody detecting phosphorylated proteins in T-cell lysates after incubation with anti-CD3ε antibody-coated beads. A longer exposure was used to show bands (arrowheads at left) migrating as p95 and p36 shown below. Arrows at the right indicate the positions of molecular mass markers. **g**, immunoprecipitation of Zap-70 and western blotting for phosphotyrosine (pY) to detect Zap-70 and CD3ζ (values below are CD3ζ ratio P23/P21).

consequently, signal transduction and microfilament re-organization.

The larger size of Mgat5-modified glycans may limit the geometry and spacing of TCR clusters in the plane of the membrane²⁴. Alternatively, Mgat5-modified glycans may bind cell-surface galectins, which restrict TCR mobility and thereby antigen-induced TCR clustering. The galectins are a widely expressed family of mammalian lectins defined as *N*-acetyllactosamine-binding proteins. The poly *N*-acetyllactosamine sequences preferentially added to Mgat5-modified glycans⁶ enhance the affinity for galectin binding (Fig. 1a). Galectins bind to lactosamine and lactose with dissociation constants in the 10^{-4} M range^{7,8}, an affinity comparable to MHC/peptide-induced oligomerization of TCRs in solution²². Therefore, the avidity of a multivalent galectin–Mgat5 glycoprotein lattice at the cell surface may be sufficient to restrict TCR clustering. To probe for the presence of galectin–glycoprotein interactions, wild-type *ex vivo* T cells were preincubated with various disaccharides for 20 min before a 10 min stimulation with anti-CD3 ϵ antibody-coated beads. Preincubation with lactose increased TCR clustering at the bead interface and reduced TCR density elsewhere on the cells (Fig. 4c), which is similar to the behaviour of untreated *Mgat5*^{−/−} T cells (Fig. 3a, b). TCR clustering was not altered by preincubation with the control disaccharide sucrose (Fig. 4b). Lactosamine and lactose both enhanced protein phosphorylation induced by anti-CD3 ϵ antibody-coated beads, but sucrose and maltose had no effect

(Fig. 4d; and data not shown). Lactose did not enhance signalling in *Mgat5*^{−/−} T cells (data not shown).

Galectin-3 was detected on the surface of naïve T cells by labelling with sulphosuccinimidobiotin, capture with streptavidin beads and western blotting with anti-galectin-3 antibodies (Fig. 4e). Chemical crosslinking of the cell surface to stabilize complexes followed by western blotting of galectin-3 immunoprecipitates showed that galectin-3 is associated with TCR complex proteins. This interaction was disrupted by either Mgat5 deficiency or incubating wild-type T cells with 2 mM lactose (Fig. 4e). Taken together, the data demonstrate that a multivalent cell-surface galectin–glycoprotein lattice limits TCR clustering in response to agonist, the avidity of which is dependent on Mgat5-modified glycans (Fig. 4f). The full complement of glycoproteins and lectins present in the T-cell lattice remains unknown, but at a minimum includes galectin-3 and the TCR complex. Exogenously added galectin-1 binds CD2, CD3, CD4, CD7, CD43 and CD45, and these proteins may also participate in the lattice²⁵. Indeed, exogenous galectin-1 modulates T-cell activation *in vitro*^{9,25}, antagonizes TCR signalling²⁶, and when injected into mice it suppresses the pathology of EAE²⁷.

The gene replacement vector used to produce our *Mgat5*-deficient mice contained the reporter gene *LacZ*, replacing the first exon, which was expressed with the same tissue specificity as *Mgat5* transcript¹⁶. Both the *LacZ* expression and cell-surface Mgat5-modified glycans in *Mgat5*^{−/−} and *Mgat5*^{+/+} T cells, respectively,

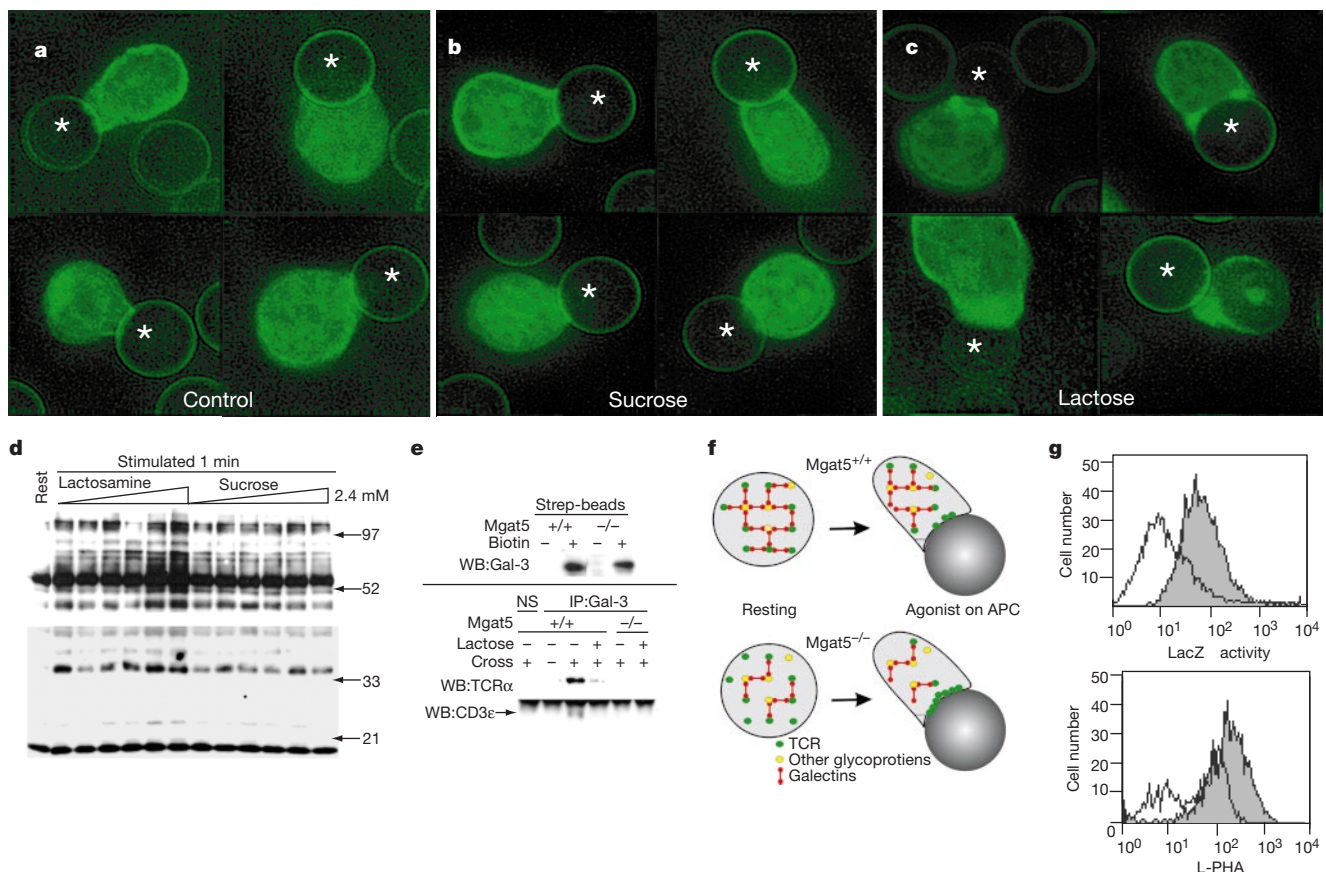


Figure 4 Lactose stimulates TCR aggregation and signalling in *Mgat5*^{+/+} mice. **a–c**, Purified T cells preincubated for 20 min with buffer (**a**), 2 mM sucrose (**b**), or 2 mM lactose (**c**), then stimulated with anti-CD3 ϵ antibody-coated beads for 10 min and stained for TCRs. Enhanced TCR clustering was observed in 0 of 10, 1 of 10 and 9 of 10 cases, respectively. **d**, *Mgat5*^{+/+} T cells incubated with increasing concentrations of disaccharide (1/3 serial dilution from 2.4 mM) and stimulated for 1 min with anti-CD3 ϵ antibody-coated beads compared for phosphotyrosine. Arrows at the right indicate the positions of molecular mass markers. A longer exposure of the lower molecular weight portion of the

blot is shown. **e**, Galectin-3 detected by surface labelling with NHS-biotin on T cells. Association of galectin-3 with CD3 ϵ and TCR α chain, and its disruption by Mgat5 deficiency and lactose is shown (bottom). **f**, Model showing restricted mobility of TCRs by interaction with a galectin–glycoprotein network, which is stronger in Mgat5-expressing cells. **g**, Upper panel shows *LacZ* activity in untreated (white) and anti-CD3 and anti-CD28-stimulated (grey) T cells from *Mgat5*^{−/−} mice. Lower panel, L-PHA-binding to *Mgat5*^{+/+} T lymphocytes either untreated (white) or stimulated with anti-CD3 and anti-CD28 for 48 h (grey).

increased 48 h after stimulation, demonstrating regulation of Mgat5 by transcriptional means (Fig. 4g). This suggests that Mgat5 enzyme activity and glycan production are limiting in resting T cells, and with stimulation, increases in Mgat5-modified glycans and galectins may dampen TCR sensitivity to antigen. Negative feedback by Mgat5-modified glycans on TCR sensitivity is delayed as it requires Mgat5 gene expression, which is dependent on T-cell activation status, and only indirectly on antigen concentrations. This form of slow-negative regulation governed by steady-state activity of the system is a key feature of robust and adaptive biochemical pathways²⁸, and Mgat5-modified glycans may contribute this feature to T-cell regulation.

It has been estimated that sustained clustering of roughly 8,000 TCRs is required for T-cell activation², but other molecular interactions clearly alter this threshold. With CD28 co-stimulation, only about 1,500 TCRs are required². Co-signalling through CD28 decreases the extent of TCR clustering needed for activation predominantly by recruiting protein kinase-enriched GM1 ganglioside rafts to the site of TCR engagement, thereby amplifying signalling^{3,5}. We show that Mgat5 deficiency increases the number of TCRs recruited to the antigen-presenting surface, thereby reducing the requirement for CD28 co-receptor engagement. This may lead to T-cell activation in the absence of CD28 co-signalling, failure of anergy and loss of immune tolerance. *CD28*^{-/-} mice are resistant to induction of EAE by low dose MBP, whereas *Mgat5*^{-/-} mice are hypersensitive, but both mutants develop clinical signs of EAE comparable to wild-type littermates with high doses of MBP²⁹. In this regard, CD28 and Mgat5 function as opposing regulators of T-cell activation thresholds and susceptibility to autoimmune disease. Mgat5-dependent glycosylation limits agonist-induced TCR clustering by sequestering receptors in a cell-surface galectin–glycoprotein lattice. However, the glycosylation deficiency in *Mgat5*^{-/-} mice affects other pathways and cell types that may also contribute to the observed autoimmunity. Indeed, Mgat5-modified glycans also reduce clusters of fibronectin receptors, causing accelerated focal adhesion turnover in fibroblasts and tumour cells; a functionality that may affect leukocyte motility¹⁶. Finally, glycosylation of Notch receptor by Fringe, a fucose-specific β 1,3-GlcNAc-transferase, provides another example of regulation by differential receptor glycosylation³⁰. In a broad context, our results suggest a general mechanism for the regulation of receptor clustering through differential glycosylation and interaction with cell-surface lectins. □

Methods

Delayed-type hypersensitivity skin reaction

To induce DTH, 100 μ l of 5% (w/v) 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) (Sigma) in ethanol/acetone (3:1, v/v) was injected epicutaneously to the shaved backs of the 129 mice. Four days after sensitization, 25 μ l of 1% (w/v) oxazolone was applied on each side of the right ear, and the left ear received 25 μ l of olive oil/acetone on each side. Ear swelling was measured with a micrometer at 24 h intervals for the next 5 d, and swelling was reported as the difference between the ear thickness of the right minus the left ears.

EAE model

Mice (129) 8–12 weeks of age were injected subcutaneously with 100 μ l of rabbit MBP (Sigma) emulsified 1:1 with complete Freund's adjuvant at three different total doses (25, 100 and 500 μ g per mouse). Mice were observed from day 5 to day 50, and observations were blinded with respect to the genotype until day 36. For lower doses of 25 and 50 μ g, half the total was injected on day 0 in the right flank and the other half on day 7 in the left flank. The high dose of 500 μ g per mouse was injected all on day 0 at the base of the tail, and 500 ng of pertussis toxin was injected through the tail vein on day 0 and day 2.

T-cell proliferation

Naïve T-cells were purified from spleens of 8–12-week-old mice by negative selection using CD3⁺ T-cell purification columns. T-cell proliferation was measured by culturing cells for 48 h in RPMI, 10% FCS, 10⁻⁵ M 2-mercaptoethanol in the presence of one or more of the following soluble antibodies: hamster anti-mouse CD3 ϵ (clone 2C11; Cedarlane), hamster anti-mouse TCR α / β (clone H59.72; Pharmingen) or 0.5 μ g ml⁻¹ anti-mouse CD 28 (Pharmingen). TPA (10 ng ml⁻¹) and ionomycin (0.5 μ g ml⁻¹) were also used to

stimulate cells. We added 2 μ Ci of [³H]thymidine for the last 20 h of incubation, and we collected cells on fibreglass filters and measured radioactivity in a β -counter.

TCR clustering

Six-micro polystyrene beads (Polysciences) in PBS were coated with hamster anti-mouse CD3 ϵ antibody (Clone 2C11; Cedarlane) at 2 μ g ml⁻¹ antibody followed by coating with 200 μ g ml⁻¹ bovine serum albumin. To measure TCR clustering, 5 $\times$ 10⁵ T cells were incubated with 2.5 $\times$ 10⁵ anti-CD3 ϵ antibody-coated beads in 100 μ l RPMI 1640 + 10% FCS at 37 °C for 10 min, placed on poly-L-lysine-coated cover slips. The cells were fixed with 10% formalin, stained with 2 μ g ml⁻¹ fluorescein isothiocyanate (FITC)-labelled anti-TCR α / β antibody (Pharmingen), solubilized with 0.2% Triton X-100, labelled with rhodamine-phalloidin and Hoechst, and then visualized by deconvolution microscopy. To measure TCR internalization, purified splenic T cells stimulated with either 0.1 μ g ml⁻¹ anti-CD3 antibody or with 10 ng ml⁻¹ TPA for varying lengths of time were collected and stained with FITC-anti-TCR α / β . TPA concentrations were not limiting as 10, 50 and 100 ng ml⁻¹ produced similar internalization and cell activation results. To measure actin reorganization, purified splenic T cells stimulated with 0.1 μ g ml⁻¹ anti-CD3 for varying lengths of time were fixed with 4% paraformaldehyde for 10 min, washed with PBS and stained with rhodamine-phalloidin, and mean fluorescence intensity (MFI) was determined by FACS.

TCR signalling

T cells (10⁶) and anti-CD3 ϵ antibody-coated beads (5 $\times$ 10⁶ at 0.4 μ g ml⁻¹ antibody) in 100 μ l RPMI 1640 were pelleted, incubated at 37 °C for various times, then solubilized with ice-cold 50 mM Tris pH 7.2, 300 mM NaCl, 0.5% Triton X-100, protease inhibitor cocktail (Boehringer Mannheim) and 2 mM orthovanadate. Zap-70 was immunoprecipitated by incubating whole-cell lysates with rabbit polyclonal anti-Zap-70 agarose conjugate (Santa Cruz) overnight at 4 °C, followed by one wash with lysis buffer and three washes with PBS. Western blotting was done with whole-cell lysates or immunoprecipitates separated on SDS–polyacrylamide gel electrophoresis gels under reducing conditions, transferred electrophoretically to polyvinylidene difluoride membranes, and immunoblotted with antibodies to Akt/PKB (NEB), phospho-Akt/PKB (NEB), phosphotyrosine (clone 4G10; Upstate Biotechnology), Zap-70 (clone Zap-70-6F7; Zymed), TCR α (polyclonal; Santa Cruz) and rabbit anti-galectin-3 (a gift from A. Raz, University of Michigan). Cell-surface proteins were biotinylated using sulphasuccinimidobiotin (NHS-biotin) for 30 min, PBS pH 8.0. Cells were lysed and labelled protein was captured on streptavidin-agarose beads. To crosslink surface proteins on purified naïve T cells, the homobifunctional crosslinker dithio-bis(sulphosuccinimidylpropionate) (DTSSP) was used at 0.1 mg ml⁻¹ with 10⁶ cell per ml in PBS pH 8.0 for 10 min at 20 °C. T cells were preincubated for 20 min with or without 2 mM lactose, and reacted with DTSSP in the presence of the same. Aliquots of cell lysate were immunoprecipitated with rabbit anti-galectin-3 antibody or non-immune rabbit serum (NS), separated on reducing SDS–PAGE, and western blotted for CD3 ϵ and TCR α chain. The band above CD3 ϵ is a cross-reactivity of secondary antibody with light-chain.

To measure Ca²⁺ mobilization, purified T cells were loaded with 10 μ M AM ester of Fluo-3 (Molecular Probes), washed and stimulated with 10 μ g ml⁻¹ anti-CD3 ϵ antibody at 37 °C. We took emission at 525 nm using a spectrofluorimeter with excitation at 488 nm. Data is plotted as a fraction of the Ca²⁺ mobilized by addition of 2 μ g ml⁻¹ of ionomycin. LacZ activity in *Mgat5*^{-/-} T cells was detected by loading cells with fluorescein-di- β -D-galactopyranoside (FDG) (Molecular Probes) at 10 °C, and allowing the reaction to proceed for 30 min. The reaction was stopped by the addition of 1 mM phenyl- β -thiogalactoside.

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Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution

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Oxygenic photosynthesis is the principal energy converter on earth. It is driven by photosystems I and II, two large protein-cofactor complexes located in the thylakoid membrane and acting in series. In photosystem II, water is oxidized; this event provides the overall process with the necessary electrons and protons, and the atmosphere with oxygen. To date, structural information on the architecture of the complex has been provided by electron microscopy of intact, active photosystem II at 15–30 Å resolution¹, and by electron crystallography on two-dimensional crystals of D1-D2-CP47 photosystem II fragments without water oxidizing activity at 8 Å resolution². Here we describe the X-ray structure of

photosystem II on the basis of crystals fully active in water oxidation³. The structure shows how protein subunits and cofactors are spatially organized. The larger subunits are assigned and the locations and orientations of the cofactors are defined. We also provide new information on the position, size and shape of the manganese cluster, which catalyzes water oxidation.

Conversion of light to chemical energy at photosystem II (PSII) is associated with charge separation across the thylakoid membrane (for review see ref. 4). It is initiated by ejection of an electron from the excited primary donor P680, a chlorophyll *a* located towards the luminal side of the membrane at the heart of the PSII reaction centre that is formed by protein subunits D1 and D2. When the cationic radical P680^{•+} is formed, the electron moves by means of a pheophytin to the electron stabilizing acceptor Q_A, a plastoquinone that is tightly bound at the stromal side of subunit D2. After each of four successive charge separating steps that are light induced, P680^{•+} abstracts one electron from a manganese cluster (generally assumed to contain four manganese ions) by means of the redox-active tyrosine residue Tyr_Z. In turn, the four positive charges accumulated in the manganese cluster oxidize two water molecules, coupled with the release of one O₂ and four H⁺. In the first two charge separations, Q_A doubly reduces one mobile molecule Q_B docked to the binding site B on D1. After uptake of two protons, Q_BH₂ is released into the plastoquinone pool that is embedded in the membrane, and replaced by a new Q_B from the pool for another round of reduction and release.

We isolated PSII from the thermophilic cyanobacterium *Synechococcus elongatus* in the form of homodimers as shown by electron microscopy (E. J. Boekema, unpublished observations). With these preparations, three-dimensional crystals were grown⁵ that are suitable for X-ray analysis (see Methods). According to SDS-polyacrylamide gel electrophoresis and mass-spectrometry (MALDI-TOF) (data not shown), this PSII is composed of at least 17 subunits⁶ of which 14 are located within the photosynthetic membrane: the reaction centre proteins D1 (PsbA) and D2 (PsbD); the chlorophyll-containing inner-antenna subunits CP43 (PsbC) and CP47 (PsbB); α- and β-subunits of cytochrome *b*-559 (PsbE and PsbF); and the smaller subunits PsbH, PsbI, PsbJ, PsbK, PsbL, PsbM, PsbN and PsbX. The membrane-extrinsic cytochrome *c*-550

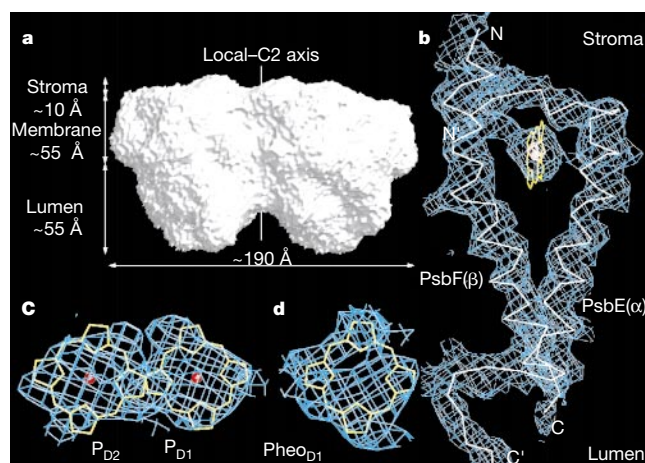


Figure 1 Electron densities of PSII after density modification and their interpretation. **a**, Surface of PSII homodimer drawn from the averaging mask generated during density modification. View direction along the membrane plane; the position of local-C2 rotation axis, is shown. **b**, Cytochrome (Cyt) *b*-559 heterodimer with electron densities contoured at 1.2 σ (r.m.s. deviation above the mean electron density) for protein and haem group, and at 4.0 σ for Fe²⁺. The termini of the α-helices are labelled N, C for the α-subunit, and N', C' for the β-subunit. **c**, Head groups of P680 chlorophyll (Chl) *a* P_{D1} and P_{D2} with view perpendicular to their planes. Mg²⁺ are depicted as red spheres. **d**, Head group of pheophytin Pheo_{D1}.

(PsbV), the protein with a relative molecular mass of 12,000 (M_r 12K) (PsbU) and the manganese-stabilizing 33K protein (PsbO) are located at the luminal side of PSII (ref. 6).

In the crystal structure, PSII occurs as a homodimer with the longest dimensions of the membrane integral part $190 \text{ \AA} \times 100 \text{ \AA}$ (Fig. 1a). This part is $40 \text{ \AA}$ thick and extends from the stromal side of the membrane by no more than $10 \text{ \AA}$. The luminal side of each monomer has prominent protrusions up to $55 \text{ \AA}$ above the membrane. The two monomers in the dimer are related by a local-C2 rotation axis orientated perpendicular to the membrane plane.

In the electron density (see Methods), we used four prominent maxima as landmarks for positioning of the individual subunits; the three smaller maxima were assigned to iron and one more extended to the manganese cluster (Fig. 2a, b, arrows). Secondary structure elements, such as α -helices and β -sheets as well as cofactors, have been modelled into the electron density map using C_α traces and porphyrin molecules. We used porphyrin molecules because the orientation in space of the tetrapyrrole planes of the chlorophyll *a* molecules, pheophytins and haems is well defined in the electron density map, whereas the orientations within the planes are arbitrary at this level of resolution. Representative electron densities are shown in Fig. 1b–d.

The membrane integral part of the PSII monomer defines the membrane plane, which is parallel to the paper plane in Fig. 2a. It is characterized by a field of 36 transmembrane α -helices. Twenty-two of these, assigned to D1, D2, CP43 and CP47, are arranged with a local pseudo-two-fold rotation symmetry. The corresponding symmetry axis, named pseudo-C2 axis, is orientated parallel to the local-C2 axis and passes through the centre of the field of α -helices and through the non-haem iron located at the stromal side of the membrane (Fig. 3a).

Two groups of five transmembrane α -helices each are arranged in two semicircles interlocked in a handshake motif and related by the pseudo-C2 axis. They have been assigned to α -helices A–E of D1 and D2, respectively, with α -helices C and D connected in each of the subunits by a long α -helix CD on the luminal side; the short α -helix DE located on the stromal side connects α -helices D and E, as proposed for PSII (refs 7, 8). Subunits D1 and D2 are clearly distinguished from each other by the position of the manganese cluster, which is coordinated by D1 (ref. 6). This assignment is supported by the location of the Q_A binding site at D2 (refs 6–8) (see below).

The arrangement of α -helices of D1 and D2 resembles that of subunits L and M in the purple bacterial reaction centre (PbRC)⁹ and the five carboxy-terminal helices of PsaA and PsaB in photosystem I (PSI) (ref. 10). This supports the hypothesis of the evolution of all photosynthetic reaction centres from one common ancestor^{2,10}. Crosslinking studies have shown that the homologous inner-antenna subunits CP43 and CP47 flank both sides of the complex D1/D2/Cyt *b*-559 (ref. 11). As D1 and CP43 are crosslinked by acceptor-side photoinhibition of PSII (ref. 12), CP43 and CP47 could be assigned. Both consist of six transmembrane α -helices arranged as a trimer of dimers related by the pseudo-C2 axis and coordinating the antenna chlorophyll *a* (see Fig. 2a). They are of similar structure to the six amino-terminal transmembrane α -helices of PsaA and PsaB in PSI (refs 2, 10). The arrangement of transmembrane helices as well as the assignment of the subunits D1, D2 and CP47 corresponds to the structural model provided by electron cryo-crystallography at $8 \text{ \AA}$ resolution of a PSII fragment^{2,13}.

One cytochrome *b*-559 has been identified in the electron density by virtue of its haeme Fe^{2+} ; it is found in the same position as proposed in ref. 13. The two subunits α and β , each forming a single transmembrane α -helix, can be distinguished because the C-terminus of the longer α -subunit extends far into the lumen (Fig. 1b). We confirmed the presence of only one cytochrome *b*-559 per reaction centre by spectroscopic analysis of fresh PSII

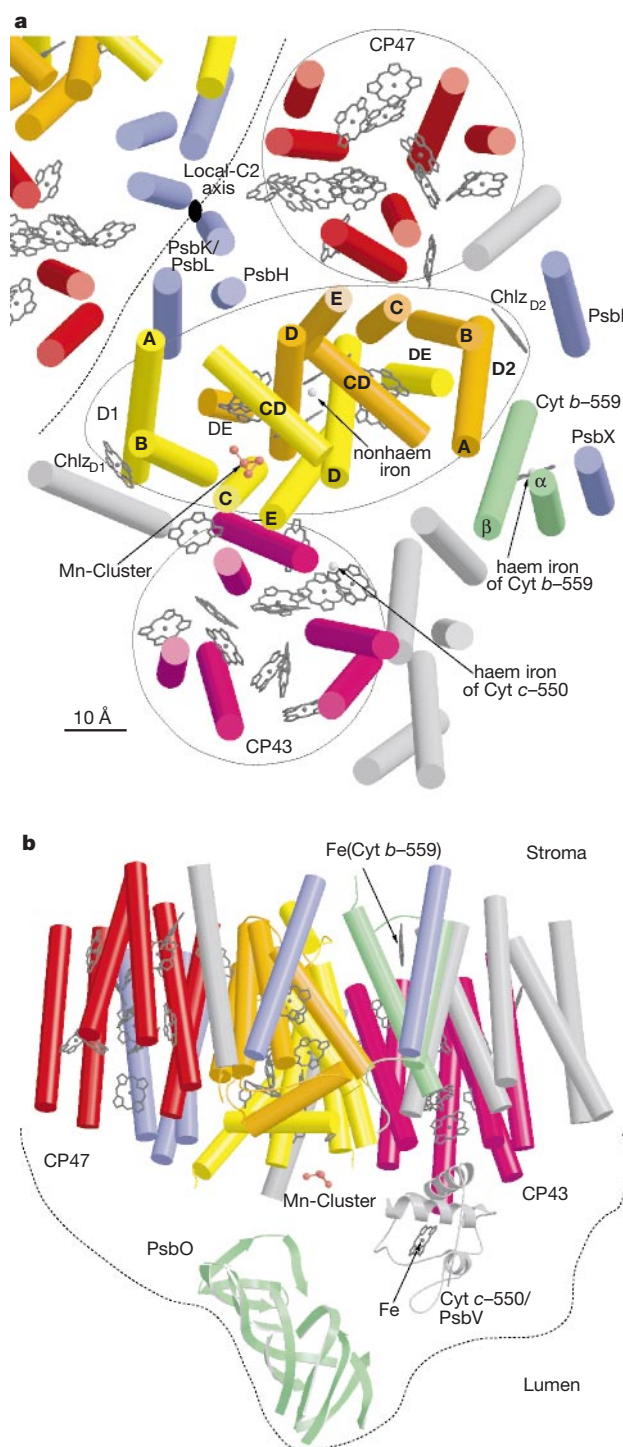


Figure 2 Structure of PSII with assignment of protein subunits and cofactors. **a**, Arrangement of transmembrane α -helices and cofactors in PSII. One monomer of the dimer is shown completely, with part of the second monomer related by the local-C2 axis (filled ellipse on the dotted interface). Chl *a* head groups and haems are indicated by black wire drawings. The view direction is from the luminal side, perpendicular to the membrane plane. The α -helices of D1, D2 and Cyt *b*-559 are labelled. D1/D2 are highlighted by an ellipse and antennae, and CP43 and CP47 by circles. Seven unassigned α -helices are shown in grey. The four prominent landmarks (three irons and the manganese (Mn) cluster) are indicated by arrows. **b**, Side view of PSII monomer looking down the long axis of the D1/D2 subunits from the right side in Fig. 2a, at slightly tilted membrane plane and rotated 180° so that the luminal side is bottom. PsbO (33K protein) is shown as a β -sheet structure (green), and Cyt *c*-550 as a helical model (grey).

preparations and of redissolved PSII crystals (data not shown). The controversial issue of one or two cytochrome *b*-559 per reaction centre present in PSII is, however, still unresolved, as this may depend on the preparation and/or organism⁶.

Crosslinking studies indicate that PsbI and PsbX are close to D2 and cytochrome *b*-559 (refs 14, 15); our electron density shows two transmembrane α -helices in this location (Fig. 2a). They could be distinguished with reference to the two-dimensional crystal structure of the D1-D2-CP47 PSII fragment^{2,13}, as it contains PsbI (and no PsbX). One unassigned α -helix² is in a position close to CP47 and D2, and was consequently assigned as PsbI (see Fig. 2a).

PsbH, PsbK and PsbL have been implicated in the stabilization of the PSII dimer^{16,17}. They were tentatively assigned to a three-helix bundle close to the local-C2 axis (Fig. 2a). In the present model, one of these three transmembrane helices is only about 13 Å apart from Q_A and could correspond to PsbH, which is supposed to influence the electron transport between Q_A and Q_B (ref. 18). Seven additional transmembrane α -helices have been found in the electron density map (Fig. 2a, b, grey α -helices) but could not be assigned.

At the luminal side of the electron density, two of the three extrinsic subunits (cytochrome *c*-550, 33K and 12K proteins) could be located unambiguously (Fig. 2b). The position of cytochrome *c*-550 is indicated by its haem iron, surrounded by three tubular structures. These helped to model part of the sequentially homologous horse heart cytochrome *c* (Protein Data Bank accession number 1HRC) into the electron density. A roughly 35 Å long, cylindrical arrangement characterized by β -strands with uncertain connectivities has been assigned to the 33K subunit (PsbO), in agreement with Fourier transform infrared data¹⁹. The modelled cylindrical structure of PsbO is tilted against the membrane by 45° and corresponds to about half of the molecular mass of this subunit. The expected extrinsic 12K subunit could not yet be located because of the high amount of non-assigned secondary structure elements belonging either to loop regions of membrane intrinsic subunits (especially the long luminal loops of CP43 and CP47) or to the remaining parts of subunit PsbO or PsbV.

The internal-antenna subunits CP43 and CP47 (Fig. 2a) contain 12 and 14 chlorophyll *a*, respectively, located in the open space between the three helix dimers, similar to that found for CP47 (ref. 2). The number of chlorophyll *a* molecules found here (26) is close to the lower limit of the range (25–50)²⁰. The chlorophyll *a* molecules in each subunit occupy two layers close to the stromal and the luminal sides of the membrane, respectively (data not shown), which is consistent with the observation that most of the conserved histidines (12 in CP43 and 8 in CP47) are also located towards the stromal and luminal ends of the respective transmembrane helices⁶. Within the two antenna systems the centre-to-centre distances between pairs of nearest chlorophylls are in the range 8.5–13.5 Å.

The cofactors of the electron transfer chain form two branches organized symmetrically along the pseudo-C2 axis (Fig. 3a, b). They were assigned to the D1 and D2 proteins by analogy with the arrangement of α -helices in L/M subunits of the PbRC. Towards the luminal side, two chlorophyll *a* molecules P_{D1} and P_{D2} (see Fig. 1c for electron density) are observed with a Mg–Mg distance of 10 Å (roughly 11 Å was assessed in ref. 2). Figure 3b shows that the head groups are parallel (with 5 Å interplanar distance) and arranged perpendicular to the membrane plane. They possibly represent P680. Owing to the large separation of these two chlorophyll molecules, excitonic coupling is weak and they can be regarded as monomeric chlorophyll molecules, suggesting that the unpaired electron in $P680^{*+}$ is located on one of them.

Towards the stromal side, two spectroscopically unidentified chlorophyll *a* molecules (Chl_{D1} and Chl_{D2}) are located at 9.8 and 10.0 Å, respectively, apart from P_{D1} and P_{D2} . Their planes are tilted roughly 30° against the membrane plane, which is analogous to the accessory bacteriochlorophylls in PbRC. They are followed by two

pheophytin molecules $Pheo_{D1}$ and $Pheo_{D2}$ (Fig. 3a). The site of tightly bound Q_A is at 12.0 Å from $Pheo_{D1}$ and occupied by a plastoquinone, the centre of its ring being at a distance of 10.5 Å from the non-haem iron. The distances between $P680^{*+}$ and Q_A , determined by electron paramagnetic resonance²¹ (27.4 ± 0.3 Å), are in agreement with the structural model ($P_{D2}-Q_A$: 28 ± 1 Å; $P_{D1}-Q_A$: 26 ± 1 Å). The putative binding site for the mobile Q_B is unoccupied, indicating that this plastoquinone probably dissociated during preparation. $P680^{*+}$ is probably located on P_{D1} , which is close to Tyr_Z , as this acts as an immediate electron donor to the cationic radical⁴.

Two extra chlorophyll *a* molecules were assigned to the spectroscopically identified species Chl_{D1} and Chl_{D2} , shown to be coordinated to His 118 in D1 and His 117 in D2 (refs 22, 23). They are 30.2 and 30.4 Å apart from P_{D1} and P_{D2} , respectively. Owing to the large separation of CP43 and CP47 from the central cofactors P_{D1} and P_{D2} of P680, a possible route for the exciton

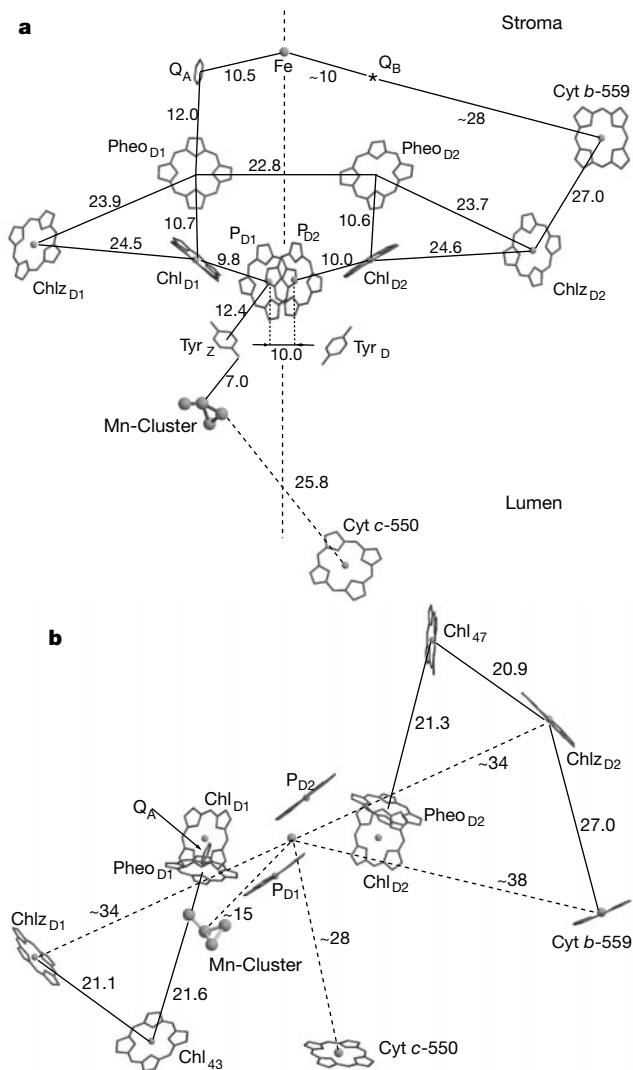


Figure 3 Arrangement of cofactors of the electron transfer chain located in subunits D1 and D2. **a**, View direction along the membrane plane. Full lines indicate centre-to-centre distances (Å) between the cofactors (uncertain to about ± 1 Å, as estimated visually). The pseudo-C2 axis is shown by the vertical dotted line; it runs through the non-haem iron Fe and is parallel to the local-C2 axis. The asterisk indicates the putative Q_B binding site. **b**, View onto membrane plane, full lines defined as in **a**, dotted lines refer to distances between centres of cofactors and the pseudo-C2 axis. As this axis is not crystallographic, distances are approximate (—). Tyrosines Tyr_Z and Tyr_D are omitted because Tyr_Z is below the manganese cluster. $Chl_{43,47}$ are the nearest Chl *a* of CP43 and CP47.

Table 1 Crystallographic statistics

Data collection	Native	Cd	Hg I	Hg II	Hg III	Au	Pt	Native
Data set*	0.934†	0.934†	0.934†	0.9072‡	0.8439§	0.9072‡	0.8439§	1.894
Wavelength (Å)	20–4.2	20–3.8	20–4.7	20–4.5	20–5.8	20–5.7	20–4.2	20–4.8
Resolution (Å)	63,639	84,964	45,429	30,210	21,643	22,458	52,611	48,420
Unique reflections	3.3	3.2	3.0	1.9	2.6	2.8	3.0	2.3
Redundancy	98.1 (98.5)	95.4 (84.5)	95.5 (87.5)	55.0 (52.1)	80.3 (82.7)	86.7 (83.9)	81.1 (81.0)	85.0 (70.7)
Completeness (%)¶	—	—	76.6 (65.8)	59.8 (53.6)	28.0 (18.7)	—	—	55.9 (44.8)
Completeness of Friedel pairs (%)¶	12.8 (3.5)#	17.1 (3.4)	14.8 (3.2)	17.5 (2.5)	11.3 (3.5)	12.5 (5.3)	13.3 (3.8)	10.5 (5.0)
$\langle I/\sigma \rangle$ ¶	6.8 (28.8)	6.8 (41.8)	4.1 (28.8)	5.4 (30.0)	4.5 (42.1)	6.2 (26.3)	5.0 (35.6)	9.2 (20.0)
R_{merge} (%)¶								
Phasing statistics								
Number of heavy atom sites	—	5	13	16	11	2	6	—
R_{cullis} (centric)	—	0.92 (0.96)	0.85 (0.85)	0.83 (0.91)	0.70 (0.83)	0.95 (0.99)	0.96 (0.99)	—
Phasing power#	—	0.78/0.49	1.07/0.73	1.21/0.91	1.12/0.75	0.62/0.44	0.49/0.51	—
Acentric/centric	—	(0.72/0.52)¶	(1.3/0.76)	(1.02/0.62)	(1.08/0.68)	(0.70/0.51)	(0.42/0.44)	—

* Heavy atoms derivatives: Cd, cadmium sulphate; Hg I, ethylmercuriophosphate; Hg II, chloromercuriacetate; Hg III, *p*-chloromercuriophenylsulphonic acid; Au, potassium dicyanoaurate (I); Pt, potassium tetracyanoplatinate(II).

† ID14-EH1 ESRF.

‡ X11 DESY.

§ BW7B DESY.

|| ID13 ESRF.

¶ The numbers in parentheses indicate the values in the highest resolution shell (taken from SCALEPACK²⁶).

Phasing power was calculated between 20 and 4.2 Å.

transfer from the antenna systems to the primary donor P680 may include the combinations Chl_{D1}/Chl_{D1} and Chl_{D2}/Chl_{D2}. As the nearest chlorophyll *a* of each antenna system in CP43 and CP47 is positioned about 21 Å apart from the respective pheophytin of the electron transfer chain (Fig. 3b), pheophytins may also be involved in exciton transfer.

The pseudo-C2 symmetry of the cofactor arrangement is broken by cytochrome *b*-559 (Figs 1b and 2a), its haem-iron being 27.0 Å apart from Chl_{D2}, and about 8 Å apart from the stromal side. For cytochrome *b*-559, different functions have been discussed⁶, including photoprotection by charge recombination between Q_B[•] and P680^{•+} (ref. 24). Such a putative pathway could include the haem of cytochrome *b*-559, Chl_{D2} and Chl_{D2} (Fig. 3a, b).

The electron transfer between P680^{•+} and the manganese cluster (see below) is bridged by the redox-active Tyr 161 of D1 (Tyr_Z). The latter could be located through a protrusion of the electron density in the last turn at the luminal side of helix C in D1. The distance from the manganese cluster is 7.0 Å (Fig. 3a). Tyr 161 of D2 (Tyr_D) is found by a corresponding electron density at helix C in D2. This position is related to that of Tyr_Z by the pseudo-C2 axis.

The centre-to-centre distance between the P680 chlorophyll

molecules and the manganese cluster is 18.5 Å (to P_{D1}) and 25.1 Å (to P_{D2}), respectively. The cluster is located on the luminal side (close to the membrane plane) and about 15 Å off the pseudo-C2 axis (Fig. 3b). The electron density of the manganese cluster is bulged in three directions in the form of a 'Y' (Fig. 4a, b).

The dimensions of the electron density contoured at the 5-σ level are 6.8 Å × 4.9 Å × 3.3 Å; the long axis (Fig. 4c) is roughly parallel to the CD helix of D1 and tilted roughly 23° against the membrane plane (Fig. 4d). In the three bulges of the electron density, three manganese ions were positioned to form the corners of an isosceles triangle, and a fourth manganese ion was placed near the centre of the triangle. The inter-atomic distances are about 3 Å, which is close to values derived from spectroscopic data²⁵. To verify that the electron density of this cluster is due to the contribution of manganese, anomalous diffraction data were collected with X-ray wavelength close to the manganese edge (1.894 Å). The global maximum of an electron density calculated with these data (not shown) fits well to the model shown in Fig. 4. In addition to manganese ions, Ca²⁺ ions are important for water oxidation; however, these could not be located at the present resolution.

The first three-dimensional structure of a water-oxidizing PSII complex described here shows the spatial distribution of most of the protein subunits and cofactors involved in excitation energy transfer and electron transport. It provides direct information regarding the size, shape and location of the manganese cluster responsible for water oxidation. The present study is an important advance and will stimulate specific functional investigations to obtain new insights into the mechanism of water oxidation by PSII.

Methods

Fully active PSII complex from the thermophilic cyanobacterium *S. elongatus* without the phycobilisome antenna was purified and crystallized as described⁵. X-ray diffraction data were collected under cryogenic conditions (100 K) at ESRF beamlines ID2B, ID13, ID14-EH1, ID14-EH2 (EMBL-outstation Grenoble, France), DESY beamlines X11, BW7B (EMBL-outstation Hamburg, Germany), BW6 (MPG-GBF, Hamburg), and Elettra beamline 5.2 (Trieste, Italy). The data were processed using DENZO²⁶ and the CCP4 program suite²⁷ to show a unit cell of dimensions $a = 130$, $b = 227$, $c = 308$ Å and space group $P2_12_12_1$. We determined phases to 4.2 Å resolution using multiple isomorphous replacement and anomalous scattering (MIRAS) within the program MLPHARE²⁷. Phase extension from 4.2 to 3.8 Å using the Cd data set was achieved by two-fold non-crystallographic averaging over the local-C2 symmetry, and solvent flattening within the program DM²⁷. We used X-ray diffraction data taken with a wavelength close to the manganese edge (see Table 1) to calculate an anomalous difference Fourier map using MIRAS phases. All electron density map visualization and interpretation was done using the program O²⁸. Figures were generated using BOBSCRIPT²⁹ and Raster3D³⁰. The current model of dimeric PSII contains 2,478 C α -positions, and two side chains of Tyr_Z, Tyr_D, 64 porphyrin molecules assigned to 64 chlorophyll *a* molecules, 4 pheophytins, 4 haems and 2 benzene molecules assigned to 2 plastoquinones, 8 manganese ions and 2 non-haem irons. With these 4,342 atoms, which represent about 10% of the total of about 45,000 atoms for the complete structural model, the crystallographic *R*-factor is 0.59.

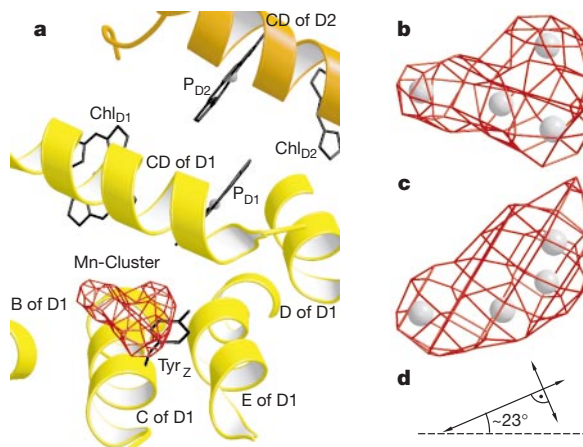


Figure 4 Location and orientation of manganese cluster. **a**, Close-up view of the reaction centre, with the electron density of the manganese cluster contoured at 5 σ. The view is from the luminal side onto the membrane plane, as in Fig. 2a. **b**, Enlarged view of the electron density of the manganese cluster; **c**, 90° rotated around the horizontal axis (view along the membrane with the luminal side on top). **d**, Orientation of the short and long axes of **c**. The latter is tilted 23° against the luminal side of the membrane plane (hatched line).

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Correspondence and requests for materials should be addressed to W.S. (e-mail: saenger@chemie.fu-berlin.de) or H.-T.W. (e-mail: witt@phosis1.chem.tu-berlin.de). Atomic coordinates are deposited in the Protein DataBank under accession number 1FE1.

corrections

Language trees support the express-train sequence of Austronesian expansion

Russell D. Gray & Fiona M. Jordan

Nature **405**, 1052–1055 (2000).

There was an error in the geographical character-state mapping in this paper. The authors inadvertently reported the values corresponding to mapping these characters onto the tree in an unordered manner. Correctly ordering the character-state changes according to the ‘express-train’ model does not change the main conclusion of the paper: the express-train model fits much better than would be expected owing to chance. The correct fit is 18 steps, and the fit of the randomly assigned character states now ranges from 95 to 122 (mean 108.4, s.d. 5.1). □

Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X₃

Veronika Souslova, Paolo Cesare, Yanning Ding, Armen N. Akopian, Louise Stanfa, Rie Suzuki, Katherine Carpenter, Daniela Nebenius-Oosthuizen, Andrew J. H. Smith, Emma J. Kidd & John N. Wood

Nature **407**, 1015–1017 (2000)

The address of the author Emma J. Kidd was cited incorrectly. Her affiliation should have been given as the Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK, which was where her work was carried out. The Welsh School of Pharmacy, Cardiff University, Cardiff CF1 3XF, UK, which was cited as her affiliation in this paper, is her present address. □

erratum

The protein–protein interaction map of *Helicobacter pylori*

Jean-Christophe Rain, Luc Selig, Hilde De Reuse, Véronique Battaglia, Céline Reverdy, Stéphane Simon, Gerlinde Lenzen, Fabien Petel, Jérôme Wojcik, Vincent Schächter, Y. Chemama, Agnès Labigne & Pierre Legrain

Nature **409**, 211–215 (2001).

The list of interactions between *Helicobacter pylori* proteins that are described and analysed in this paper are available as Supplementary Information on Nature’s World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of *Nature*. Proteins are named according to the nomenclature of The Institute for Genomic Research microbial database. □

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